Occurrence, Survival, and Characterization of *Legionella* in Water

by

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ABSTRACT

Bacteria of the *Legionella* genus are a water-borne pathogen of increasing concern due to being responsible for more annual drinking water related disease outbreaks in the United States than all other microbes combined. Unfortunately, the development of public health policies concerning *Legionella* has been impeded by several key factors, including a paucity of data on their interactions and growth requirements in water distribution networks, a poor understanding of potential transmission sources for legionellosis, and limitations in current methodology for the characterization of these pathogens. To address these issues, a variety of research approaches were taken. By measuring *Legionella* survival in tap water, association in pipe material biofilms, population dynamics in a model distribution system, and occurrence in drinking water distribution system biofilms, key aspects of *Legionella* ecology in drinking water systems were revealed. Through a series of experiments qualitatively and quantitatively examining the growth of *Legionella* via nutrients obtained from several water sources, environmental nutritional requirements and capability for growth in the absence of host organisms were demonstrated. An examination of automobile windshield washer fluid as a possible source of legionellosis transmission revealed *Legionella* survival in certain windshield washer fluids, growth within washer fluid reservoirs, high levels and frequency of contamination in washer fluid reservoirs, and the presence of viable cells in washer fluid spray, suggesting the potential for exposure to *Legionella* from this novel source. After performing a systematic and quantitative analysis of methodology optimization for the analysis of *Legionella* cells via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, several

strains of this microbe isolated from separated and varied environmental water sampling sites were distinctly typed, demonstrating a potential application of this technology for the characterization of *Legionella*. The results from this study provide novel insight and methodology relevant to the development of programs for the monitoring and treatment of *Legionella* in drinking water systems.

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BACKGROUND

1.1 Introduction

Since the discovery as the causative agent of the potentially deadly pneumonia Legionnaires' disease, *Legionella* have become a waterborne pathogen of increasing concern. Consistently rising incidence after the first reported outbreak in 1976 (Brenner et al., 1979), frequent high profile outbreaks, and the discovery of numerous novel sources of transmission has caused these bacteria to become increasingly relevant from both a scientific and lay perspective. Unfortunately, this ubiquitously occurring water-borne environmental human pathogen to which individuals are exposed to regularly is poorly understood in many ways. Two simple facts demonstrate how this lack of information leads to potentially unnecessary public health risk: 1) *Legionella* cause more drinking water related outbreaks in America than all parasites, other bacteria, viruses, fungi, and chemicals combined (Brunkard et al., 2011), and 2) no U.S. federal or state laws mandating *Legionella* specific monitoring or treatment for public drinking water utilities exist.

There are many reasons, ranging from political to economic to scientific, why these two seemingly incongruous statements are simultaneously true. Amongst the major components responsible for the non-existence of *Legionella* regulation is a sheer lack of information about this pathogen. Unfortunately, as long as key knowledge gaps exist concerning the dynamics of how *Legionella* interact with water distribution systems, effective policies to reduce the transmission of legionellosis will be difficult to intelligently design, implement, and regulate. Without a fundamental understanding of

how these bacteria grow and survive in the environment, water treatment methods aimed at reducing or eliminating *Legionella* contamination may prove ineffective. Until all relevant reservoirs for *Legionella* are discovered, monitoring programs may overlook important, yet unknown, sources of transmission. Issues with current detection and characterization methodology for *Legionella* may translate to inaccuracies when these techniques are applied towards analysis of *Legionella* within the environment. The goal of this research was to aid in solving these and similar issues through the elucidation of important unknowns concerning *Legionella*.

1.2 Objectives

The aim of this project was to address knowledge gaps concerning the ecology and physiology of *Legionella*. Four primary objectives were addressed through research utilizing a variety of approaches:

1. to investigate occurrence and *Legionella* survival in drinking water distribution systems. This was accomplished through experiments examining the impact of temperature on *Legionella* survival in water, the effect of water pipe material on *Legionella* biofilm association, the dynamics of *Legionella* populations within a model drinking water distribution system, and a field study of *Legionella* occurrence within domestic water meters.

2. to evaluate nutritional requirements and availability for *Legionella* in water. This was accomplished through experiments detecting *Legionella* colony formation on media

supplemented with nutrients from water sources and measuring *Legionella* population dynamics in water containing nutrients from various water sources.

3. to determine potential for *Legionella* transmission from automobile windshield washer fluid. This was accomplished through experiments measuring *Legionella* survival in windshield washer fluid, determining the capability for *Legionella* growth within windshield washer fluid reservoirs, and a field study of *Legionella* occurrence in windshield washer fluid reservoirs.

4. to implement a MALDI-TOF-MS based typing method for *Legionella*. This was accomplished through experiments optimizing a MALDI-TOF-MS based characterization procedure for *Legionella* and evaluating this procedure via the examination of environmental strains of *Legionella.*

1.2 Literature Review

1.2.1 *Legionella* Physiology

Bacteria of the *Legionella* genus fall into a single family, Legionellaceae, containing over 50 species and 70 serogroups (Diederen 2008). These bacteria are heterotrophic, non-spore forming, non-encapsulated, aerobic, and Gram-negative, although lipid rich outer cell membrane leaflet result in poor Gram staining and necessitate silver staining for visualization. Cell walls contain side chains responsible for somatic antigen specificity (Thomason and Bibb 1984). *Legionella* are differentially motile, depending on their cell state, due to single polar flagella. A high degree of pleomorphicity has been demonstrated by the genus (Faulkner and Garduño 2002), similarly due to differential protein expression in response to environmental stimuli and replication cycling. Cell morphology exists in several forms including: 0.5-1.0 µm cocci, 1.0-2.5 µm bacilli, diplobacilli, and 5-50 µm filaments. A high degree of genetic variation exists amongst the genus, with numerous species-specific genetic markers existing (Diederen 2008).

Legionella are considered fastidious organisms, as they require high concentrations of soluble iron (Reeves et al., 1981) and L-cysteine (Ewann and Hoffman 2006) for growth. L-cysteine is an essential amino acids for *Legionella* due to the lack of essential cysteine biosynthesis enzymes in most strains, resulting in a nearly genus-wide auxotrophy for this compound. The high iron requirements for growth are potentially due to low-efficiency iron uptake mechanisms and a necessity for the synthesis of essential iron-containing proteins (Mengaud and Horwitz 1993). It is believe the vast majority of

these two required (and many other) nutrients are sourced directly from parasitized eukaryotic host cells or from other microbes present in biofilms *Legionella* inhabit via necrotrophy (Temmermen et al., 2006). Although high concentrations of specific nutrients are needed for replication, *Legionella* are capable of long term survival in oligotrophic conditions. This survival is often attributed to biofilm association (Diederen 2008) and may be facilitated in the environment through the conversion to cell forms with substantially reduced metabolic processes, including filaments and spore-like cocci (Faulkner et al., 2008).

A high level degree of environment dependent developmental cycling has been demonstrated for *Legionella,* resulting in the ability for these bacteria to exist in several distinct forms related to survival and replication (Garduño et al., 2002), each with vastly different forms of metabolism and protein expression. A stationary phase form expressed in the presence of potential host cells results in bacilli with flagella expression, motility, and increased host infectivity. Upon infection of a host cell or in the presence of sufficient extra-cellular nutrients, a replicative form of non-motile cocci is expressed. Upon consumption of available nutrients, a mature infectious form distinct from the stationary phase form, but also motile and infectious is produced. Intermediate forms are known to exist, as well as the aforementioned dormant cocci and filaments (Al-Bana et al., 2014). In addition to nutrient and host presence, environmental stresses such as heat and chemical disinfectants have been shown to stimulate morphological changes in *Legionella* cells (Piao et al., 2006).

1.2.2 *Legionella* ecology

Legionella are ubiquitiously found in artificial and natural fresh water sources, but have been also detected in salt and brackish environments (Gast et al., 2011). They are capable of long term survival in a wide variety of environments due to the formation of dormant cell forms, biofilm association, and host parasitization (Rowbotham 1980). An affinity for high temperatures is a hallmark of *Legionella* species, with most requiring temperatures between 20-48 °C for growth (Kusnetsoz et al., 1996, Schulze-Röbbecke et al., 1987). *Legionella* have demonstrated the capability to tolerate a much wider range of temperatures, including 50 °C for extended periods of time (Dennis et al., 1984) and short durations of up to 68 °C without substantial decreases in concentration. *Legionella* possess an ideal pH range for growth of 6.0-8.0 (Ohno et al., 2003), with survival demonstrated in extended exposure to 4.0-9.5 (Katz and Hammel 1987). Relative to other water-borne pathogens and indicator organisms, *Legionella*, particularly when associated with biofilms or infecting host organisms, demonstrate a high level of resistance to conventional water treatment methods such as chlorine (Cooper and Hanlon 2010), heat, ozone, and ultraviolet radiation (Muraca et al., 1987). This resistance has been reflected in numerous studies demonstrating the difficulty associated with eliminating *Legionella* contamination from water distribution systems (Marchesi et al., 2011).

Through a combination resistance mechanisms toward multiple forms of environmental stress, including starvation induced cell cycling, *Legionella* populations are capable of long-term survival in unfavorable conditions. Affinity for warm temperatures and biofilm association leads to heated engineered water systems often

serving as reservoirs for these pathogens (Diederen, 2008). Hot tap water systems are infamously capable of providing an ideal environment for *Legionella*, potentially due to selective pressure caused by temperature and disinfectants reducing microbial competition. In such systems, *Legionella* are capable of maintaining concentrations despite oligotrophic conditions for years (van der Kooij et al., 2005) and remain able to produce population blooms induced by favorable conditions, particularly sufficient nutrients sourced from eukaryotic hosts or other microbes entering the systems. The efficacy of methods for feasibly inhibiting growth and survival of *Legionella* in tap water systems are debated, with appropriate temperature control and pipe material often cited as possible solutions due to studies demonstrating the impact of these conditions on *Legionella* survival (Rogers et al., 1994). Unfortunately these two particular approaches are not universally effective or applicable, as temperatures above 50 °C required for *Legionella* control can pose a scalding risk in water intended for human use, and the antimicrobial effects of certain pipe materials such as copper seem to be temporary or inconsistent (Buse et al., 2014) at inhibiting the growth and survival of these pathogens.

Legionella occurrence is common in a wide variety of natural and anthropogenic water sources, with frequency and concentration varying by source, region, season, and other environmental factors. Surface water is a common reservoir for and thought to be the primary environmental habitat of *Legionella*. Numerous studies have demonstrated high positivity for *Legionella* in rivers and lakes, often with nearly universal positivity (reaching over 99%) unaffected by water chemistry parameters (Fliermans et al., 1981) and in concentrations greater than 10^3 cells/mL. While much less studied, ground water has been demonstrated to another common environmental source for *Legionella*.

Concentrations and frequency of *Legionella* in ground water tend to be lower than in surface water, although studies have demonstrated positivity over 90% (Lye et al., 1997) and concentrations greater than 10^2 colony forming units (CFU)/mL (Brooks et al., 2004). Artificial water systems are often highly contaminated with *Legionella*, presumably due to preferential environmental conditions not typically found in natural water sources, such as elevated temperature, reduced competition from disinfection, and high biofilm surface area to water volume ratio. Drinking water distribution systems are considered to be ubiquitously contaminated by *Legionella*, as demonstrated by a United States Environmental Protection Agency (USEPA) study recording *L. pneumophila* presence in nearly 50% of drinking water taps sampled across the United States, with an average concentration of 2 genomic units/mL (Donohue et al. 2014). Due to increased nutrient concentration and, often, temperatures conducive to *Legionella* growth, waste water often represents an ideal environment for *Legionella*. This has been demonstrated in several studies showing 100% positivity for contamination (Palmer et al., 1993) and high concentrations greater than 10^7 CFU/mL (Olsen et al., 2010). The high levels of *Legionella* contamination in waste water often lead to the presence of these microbes in treated waste water designed for reuse. While only a handful of studies examining *Legionella* in recycled water have been conducted, all have demonstrated positivity greater than 30% (Alonso et al., 2006). Concentrations greater than 10 CFU/mL have been detected in tertiary treated effluent (Jjemba et al, 2010) and regrowth within reclaimed water distribution systems has been established (Fahrenfeld et al., 2013). Although most commonly associated with aquatic systems, *Legionella* are capable of survival in most environments, particularly soil, and often in association with host

organisms (Rowbotham 1980). Soil borne *Legionella* occur in varying levels of frequency in soil additives for agricultural purposes, including 73% of potting soil samples (Steele et al., 1990) and concentrations as high as 10^8 CFU/g of compost (Casati et al., 2010).

1.2.3 Legionellosis

The first reported outbreak of legionellosis occurred during the summer of 1976 in Bellevue-Stratford hotel in Philadelphia, PA during an American Legion convention, in which 221 cases of pneumonia and 34 deaths occurred. After a 6 month long investigation by public health authorities, the causative was found to be a previously discovered environmental bacterium which was named *Legionella pneumophila* (Fraser et al., 1997) in honor of the Legionnaires affected and due to the organism's affinity for causing lung infection. Earlier cases and outbreaks of legionellosis have since been established, including a then unresolved outbreak of a febrile illness in Pontiac, MI in 1968, which was later confirmed to be caused by exposure to *Legionella* (Glick et al., 1977). *L. pneumophila* is the most commonly reported species responsible of legionellosis, responsible for approximately 90% of cases (Yu et al., 2002), followed by *L micdadei, L. bozemanii, L. dumoffii,* and *L. lonbeachae*. Of the reported Legionnaires' disease cases caused by *L. pneumophila*, nearly 70% are due to strains belonging to Serogroup 1 (Joseph 2004), highlighting the reason for the emphasis placed on this particular strain and species in health related *Legionella* research. Incidence of legionellosis has consistently and substantially increased since the discovery of the disease (Hicks et al., 2011), with *Legionella* currently being responsible for more

drinking water related disease outbreaks in the United States than all other microbes and chemicals combined, leading to the recent addition of *Legionella pneumophila* on the USEPA contaminant candidate list in 2009 (Brunkard et al., 2011). Although federal, state, municipal, and industry guidelines for legionellosis prevention exist (Parr et al., 2014) (including a USEPA Safe Drinking Water Act stipulation that water properly treated for *Cryptosporidium*, *Giardia*, and viruses will most likely have negligible levels of *Legionella*), no regulations specific for *Legionella* outside of the local level are in place in the United States. This will change in the near future, as a document outlining minimum legionellosis risk management guidelines for building water systems is currently being drafted by the American Society of Heating, Refrigerating, and Air-Conditioning Engineers. The anticipated release of these guidelines in 2015 will mark the first major national regulatory standards specific for *Legionella* monitoring and treatment in the United States, nearly 40 years after the discovery of Legionnaires' disease.

Legionellosis occurs in two primary forms: Pontiac fever and Legionnaires' disease. Pontiac fever is a self-limiting febrile disease caused by infection of epithelial cells and macrophages located in the upper respiratory tract (Glick et al. 1977). Symptoms typically occur within 3 days of infection and last for 2-5 days, although the disease is rarely fatal and treatment is normally supportive. Pontiac fever is extremely underdiagnosed and very rarely reported outside of outbreaks (Pancer and Stypulkowska-Misiurewicz, 2003), but is predicted to be the most common form of legionellosis, causing up to 95% of cases. Legionnaires' disease is a potentially deadly pneumonia caused by infection of alveolar macrophages and epithelial cells in the lower respiratory

tract (McDade et al., 1977). Symptoms occur within 2-10 days of infection and last for 7-10 days. Fatality rates for both sporadic and outbreak cases vary drastically, between 1% and 80% dependent on factors such as treatment, underlying health issues, causative strain, and source of infection (Benin et al., 2002). Diagnosis of legionellosis is typically performed via urine antigen analysis (Edelstein 2007) and isolation (Den Boer and Yzerman, 2004). Although Pontiac fever is often not substantially linked to any risk factors, several major ones exist for Legionnaires' disease (Fields et al., 2002). These risk factors generally coincide with those for other forms of typical pneumonia and include smoking, old age, male gender, immunodeficiency, recent surgical operation, and throat obstructions.

Transmission of legionellosis occurs due to environmental exposure from a source of water, air, or soil contaminated with *Legionella*. To date, there have been no reported cases of human-to-human transmission of legionellosis (Stout and Yu, 1997). The two primary routes of transmission are aspiration and inhalation (Yu 1993). Aspiration of microdroplets of water entering mouth during the act of drinking water, a process which is a common cause of general pneumonias, most frequently occurs in bed-ridden individuals with some form of throat obstruction. Inhalation of aerosolized *Legionella* cells or microdroplets of water can also occur during respiration (Fraser et al., 1977) and is commonly accepted to be the primary route of transmission for legionellosis, though this is debatable as aspiration is often ignored as a possible route during epidemiological studies. The primary route of transmission from soil-borne cases has not been established; hand-to-mouth transmission has oddly been suggested (most likely due to this route being common for other soil-borne pathogens), although inhalation of

bioaerosols containing *Legionella* is most likely (Conza et al., 2013). Both forms of transmission for Legionnaires' disease require *Legionella* cells or microdroplets within a particular size range to enter the lungs and cause infection, with particles ranging from 1-10 µm in diameter able to effectively enter and deposit within alveola (Schoen and Ashbolt, 2011). Non-respiratory forms of legionellosis, most often caused by non-*pneumophila* species, have been documented with increasing frequency in recent years. The most common forms of atypical legionellosis are cellulitis/faciitis (Kilborn et al., 1992) and endocarditis (McCabe et al., 1984), although other presentations, including several cases of central nervous system cases have been recently reported (Perpoint et al., 2013). A widely accepted mean infectious dose of *Legionella* required to cause Legionnaires' disease in healthy individuals has yet to be established (Delgado-Viscogliosi et al., 2005), with estimates between 1-100,000 cells deposited in the lungs necessary to cause infection. A major limiting fact in the discernment of an infectious dose is the fact that *Legionella* infectivity is highly dependent on strain and cell-cycling dependent virulence of transmitted cells. The overwhelmingly vast majority (>95%) of reported legionellosis cases in the United States are traced to contaminated drinking water sources (Brunkard et al., 2011), although soil-borne transmission have become increasingly common in other countries (Currie et al., 2014) and is even responsible for the majority of cases in New Zealand.

1.2.4 *Legionella* identification and characterization

Detection and quantification of *Legionella* in environmental water, air, and soil samples is typically performed via culturing, PCR, or direct microscopy. Culturing is

typically performed on Buffered Charcoal Yeast Extract (BCYE) agar medium (CDC 2005), containing yeast extract (for carbon and other nutrients), ACES buffer (to maintain pH), charcoal (to bind free radicals), iron (a trace nutrient), L-cysteine (a trace nutrient), and α-ketoglutarate (a supplemental trace nutrient). BCYE medium utilized for environmental samples is often supplemented with a cocktail of *Legionella* selective antibiotics, most commonly glycine (a general antibacterial), polymixin-B (for Gram-negative bacteria), cyclohexamide (for eukaryotic microbes), and vancomycin (for Gram-positive bacteria). Incubation for most species of *Legionella* is performed at 35 °C for 2-10 days. An extended heat amplification of water samples or co-culturing with amoebae can increase odds of colony formation due to resuscitation of viable but non-culturable cells. Most *Legionella* species exhibit a distinct colony morphology with a texture similar to cut glass, allowing visual confirmation, although colony color and UV fluorescence can drastically vary amongst species and strains. PCR based detection of *Legionella* is most often accomplished via amplification of *Legionella* specific 16s rRNA (Bej et al., 1991), or genus specific genes, most commonly *mip* (Ratcliff et al., 1998). Real-time PCR (Ballard et al., 2000) has become increasingly popular as a method for quantification of *Legionella* (Diederen 2008). This method is often contested as a more appropriate 'gold standard' than culturing for use in the detection of *Legionella*, particularly in environmental samples, in which *Legionella* are known to often be difficult to culture. Despite several inherent benefits, PCR has been shown to have potential drawbacks, including detection of non-viable cells or naked DNA, as well as reports of false-positive results in clinical samples (Cloud et al., 2000). Direct fluorescent-antibody microscopy using *Legionella* specific antibodies was once a popular

method for the detection and quantification of *Legionella* in environmental and clinical samples (Cordes et al., 1981). This technique has shown to be as sensitive and specific as PCR and culturing, with greater accuracy in detecting total cells present in a sample (Yamamoto et al., 1993). This form of microscopic identification and quantification of *Legionella* was once commonly used for examining environmental samples, but has fallen out of common practice outside of the medical industry, in part due to the development of simpler and lower cost methods.

Typing methods are commonly used in ecological studies of *Legionella* and epidemiological investigations of legionellosis cases. Shortly after its discovery as a common environmentally derived human pathogen, methods for the characterization of *Legionella* isolates began to be developed. One of the earliest techniques for characterization developed was serotyping (Wilkinson and Fikes 1980), which to this date remains a commonly used method for differentiation of *Legionella* strains. Serological typing of *Legionella* is particularly common in the clinical setting for use in distinguishing serogroup one *Legionella*, which are responsible for more Legionnaires' disease cases than all other serogroups combined (Donohue et al., 2014). Over the years, other methods of subgrouping have been developed for *Legionella*, including monoclonal antibody subgrouping (Watkins et al., 1985), repetitive element PCR (Georghiou et al., 1994), pulsed-field gel electrophoresis (Schoonmaker et al., 1992), and amplified fragment length polymorphism (Fry et al., 1999) typing. The current 'gold standard' for *L. pneumophila* typing revolves around an ordered seven allele sequence-based typing (SBT) scheme (Gaia et al., 2005, Ratzow et al., 2007), which currently has produced over 1880 sequence types for the genus. Improvements in deep-sequencing technology have

led to the development of whole genome sequencing (WGS) of *Legionella* subgrouping (Underwood et al., 2013), an attractive high resolution alternative as deep sequencing technology continues to fall in price. While each of these listed methods has potential for use in certain circumstances, they are all have a substantial monetary cost and lengthy assay time, limiting their applicability.

While immunological and genetic approaches have been successfully used for the typing of *Legionella* (Garcia-Nunez et al., 2013, Jarraud et al., 2013), these methods can be considered time consuming and costly, two characteristics which are significantly improved upon through the use of Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) (Kliem and Sauer 2012). MALDI-TOF-MS has been used increasingly in recent years as a tool for clinical microbiologists to efficiently identify pathogens. The approach has been proposed as an alternative to conventional methods for the typing of microbes. Studies have shown promising results for a number of organisms, particularly those of clinical relevance. *E. coli* isolates have been successfully identified based on pathotype (Clark et al., 2013), and MALDI-TOF-MS was found to be a more rapid alternative to repetitive sequence based-PCR for the typing of drug resistant *Acinetobacter* (Mencacci et al., 2013). In addition to clinical microbes, this technology has been successfully applied to the typing of environmental isolates of a number of bacteria including lactic acid bacteria, *E. coli*, and *Enterococcus* (Doan et al., 2012, Siegrist et al., 2007, Giebel et al., 2008).

Despite a large amount of work being performed on the use of MALDI-TOF-MS for the identification of *Legionella* species in clinical settings, few studies have examined *Legionella* isolated from environmental samples. Gaia et al. (2012) compared MALDI-

TOF MS data from hundreds of clinical, water, cooling tower, and soil isolates of various *Legionella* species to conventional sequence based identification. Moliner et al. (2010) demonstrated a high level of sensitivity and specificity for the use of MALDI-TOF-MS in the identification of clinical and environmental *Legionella*. Svarrer and Uldum (2011) examined the distribution of *Legionella* species in clinical and environmental isolates from Denmark using MALDI-TOF-MS. Pennanec et al. (2012) investigated the refinement of a MALDI-TOF-MS protocol for the identification of environmental bacteria, applying it successfully to *Legionella* obtained from a cooling tower. While these and other studies have advanced research on the application of MALDI-TOF-MS for *Legionella* species and genus-level identification, to date only a single published study has reported strain-level differentiation of *Legionella* (Fujinami et al., 2010) using this promising technology.

CHAPTER 2

SURVIVAL OF *LEGIONELLA* IN TAP WATER

2.1: Abstract

Legionella are an increasingly relevant yet poorly understood waterborne pathogen often associated with engineered water systems. Current information on the interactions of *Legionella* with biological and abiotic aspects of municipal water distribution systems is lacking in key areas. To address these knowledge gaps, the growth and survival of *Legionella* in water distribution systems was studied. When incubated in tap water at 4, 25, and 32 °C, *L. pneumophila* survival trends varied with temperature, with growth potentially occurring at the two latter temperatures. Stable populations were maintained for months at 25 and 32 °C demonstrating the capability of *Legionella* to survive for extended periods of times under these conditions despite a lack of biofilm development and low levels of nutrients. After inoculation of reactors containing coupons of PVC, copper, brass, and cast iron, *L. pneumophila* colonized biofilms formed on each pipe material in days, with concentrations over time on each material being similar, save for cast iron, which contained 1-log less *Legionella* after 90 days. 10^7 *L. pneumophila* cells spiked into a 50 L model water distribution system rapidly dispersed throughout and colonized the system within 3 days. In addition, *Legionella* demonstrated an apparent delayed biofilm release dynamic following growth periods. Chlorination of the system had a greater effect on biofilm associated *Legionella* concentrations, with populations in water and biofilm returning to pre-chlorination levels within 6 weeks. Biofilms sampled from residential drinking water meters collected from two general areas within central Arizona were analyzed via PCR for the presence of

Legionella and *L. pneumophila*. One area consistently produced samples containing both, with 26% and 14% positive for *Legionella* and *L. pneumophila*, respectively. A lack of any positive samples from the second area indicate environmental differences in water distribution systems may have an impact on the survival of *Legionella*. The results provided by this study document the survival of *Legionella* under different environmental conditions and provide relevant knowledge in identifying conditions which promote survival and potential growth of these pathogens.

2.2: Introduction

The *Legionella* genus contains over 50 species of gammaproteobacteria (Diederen, 2008), many of which are capable of producing respiratory illness in humans. These bacteria are ubiquitous in both natural and artificial water systems where their growth is often associated with biofilms. While not necessary for growth, biofilm habitation provides *Legionella* protection from harmful substances such as disinfectants as well as more ready access to eukaryotic host organisms important for replication in the environment (Donlan et al., 2005). The typical hosts for these bacteria are thought to be protozoan biofilm grazers (Valster et al., 2010), notably amoeba such as *Hartmanella* (Wadowsky et al., 1991). Despite this, *Legionella* are also capable of infecting a wide variety of eukaryotes, with humans being a potential incidental host. Although able to survive in a wide range of temperatures, *Legionella* typically require relatively warm environments found in anthropogenic water systems to reach population levels capable of posing public health risks to humans (Declerck et al., 2012).

The first reported outbreak of legionellosis occurred at an American Legion convention in Philadelphia, PA during the summer 1976. Several months later, the causative agent of the pneumonia cases linked to the outbreak was found to be a previously unidentified environmental bacterium, *Legionella pneumophila* (Brenner et al., 1979). While various forms of legionellosis infections have been documented, the overwhelmingly vast majority result in one of two respiratory diseases: Pontiac fever is a self-limiting febrile disease that is poorly documented, whereas Legionnaires' disease is a potentially deadly pneumonia which is often highly publicized and reported (Hicks et al., 2011). Though a majority of confirmed Legionnaires' disease cases occur in the hospital setting, both community acquired and nosocomial outbreaks of legionellosis occur regularly (Stout and Yu 1997). Drastically increasing rates of legionellosis outbreaks has led to *Legionella* being responsible for more water borne disease outbreaks in the United States than any other microbe (Brunkard et al., 2011), highlighting the importance of the development and implementation of measures to prevent this disease.

As *Legionella* require temperatures above 20 °C to grow, contamination is often associated with heated artificial water systems such as cooling towers, spas, and hot tap water lines (Farhat et al., 2012). As a result the vast majority of legionellosis prevention measures are aimed at treatment of these high temperature systems. Although the warm temperatures necessary for *Legionella* replication are more likely to occur in these environments, non-heated engineered water systems are also common sources of outbreaks (Kool et al., 1999). Due to this fact, the impact of drinking water main distribution systems on the transmission of legionellosis warrants examination. While a large number of studies have been aimed at investigating the incidence and survival of

Legionella at the in-premise level, a relatively small amount of research has been focused on *Legionella* within main distribution systems (Donohue et al., 2014), leading to a knowledge gap on their interactions with these systems. The objectives of this study were to elucidate the ecology of *Legionella* in drinking water distribution systems via the following experiments: 1) a bench scale experiment to measure the capability for *Legionella* to survive in tap water at varying temperature, 2) a bench scale experiment to determine the association and survival of *Legionella* within biofilms on various water pipe materials, 3) a pilot scale experiment examining the interactions between *Legionella* in flowing water and biofilms of a model distribution system (MDS), and 4) a field study of the occurrence of *Legionella* in drinking water distribution system biofilms from residential water meters.

2.3: Methods

2.3.1: Media and laboratory strain of *Legionella*

All *Legionella* cultures were prepared according to the methods previously described by the United States Centers for Disease Control and Prevention (CDC 2005). Culturing was performed using buffered charcoal yeast extract agar medium (BCYE) (BCYE Agar Base, Benson, Dickson, and Company, Franklin Lakes, NJ, USA). BCYE was prepared with the following supplements: 0.3% glycine, 100 units/mL polymixin B, 5 µg/mL vancomycin, and 80 µg/mL cyclohexamide, and0.4g L-cysteine HCl. *L. pnuemophila* stock cultures were grown at 37 °C with agitation over 72 hours in Buffered Yeast Extract (BYE) liquid medium. Cell concentrations in stock cultures were

estimated using optical density measurement at 600 nm. The spread plate technique was used to quantify *Legionella* after incubation at 37 °C for 72 hours, with up to 7 additional days as needed. All laboratory experiments utilized the *L. pneumophila* serogroup 1 strain Knoxville-1 (American Type Culture Collection 33153).

2.3.2: Molecular detection of *Legionella*

DNA extraction was performed on drinking water meter biofilm samples using a ZYMO Research yeast/bacterial DNA extraction kit (Zymo Research Corporation, Irvine, CA, USA). *Legionella* spp. and *L. pneumophila* specific primers were used in this study (Table 1). For both primer sets, PCR amplification mixtures consisted of: 12.5 µL Promega GoTaq Green MasterMix (Promega Biosciences LLC., San Luis Obispo, CA, USA), 10 µL DNA template, and 0.13 µM each primer, with a final reaction volume of 25 µL. Gel electrophoresis was performed in a 40 mL 1% agarose gel containing 2µL of 10,000X Invitrogen SYBR Safe DNA Gel Stain (Life Technologies Corporation, Carlsbad, CA, USA) to detect PCR products. Sequencing was performed on PCR products to confirm the presence of *Legionella* or *L. pneumophila*.

Table 1: PCR Primers used in the study

Primers	Sequences (5'→3')	Gene Amplified	Amplicon Length	Reference
LEG-226 LEG-858	AAGATTAGCCTGCGTCCGAT GTCAACTTATCGCGTTTGCT	*Legionella* 16S rRNA	654 bp	Wullings et al. 2011

LpneuF LpneuR	CCGATGCCACATCATTAGC CCAATTGAGCGCCACTCATAG	*L. pneumophila* *mip*	150 bp	Wullings et al. 2011

2.3.3: Survival of *Legionella* in tap water at 4, 25, and 32 °C

A series of 50 mL polystyrene conical tubes were filled with 50 mL of dechlorinated tap water from the city of Tempe, AZ, USA. Following inoculation with $3x10^7$ *L. pneumophila* cells, duplicate tubes were incubated at 4, 25, and 32 °C. The temperatures chosen for this experiment were intended to represent the range of temperatures commonly recorded in water distribution systems across the United States. The study parameters simulate conditions in an actual distribution system where growth of *Legionella* may be possible (25 and 32 °C) and a condition under which replication cannot occur (4 °C) (Schulze-Röbbecke et al., 1987). Over the course of 97 days, the tubes were sampled periodically and colony forming units (CFU)/mL were determined via the spread plate technique on BCYE agar medium.

2.3.4: Association of *Legionella* with biofilms on a variety of pipe coupons

A series of 5 L polyethylene containers were filled with 1 L of tap water from the city of Tempe, AZ, USA. Coupons of polyvinyl chloride (PVC), cast iron, copper, and brass were placed in the containers and incubated at 25 °C for 3 weeks to allow biofilm development. The materials studied were chosen to represent a range of pipes and water fixtures commonly found in drinking water distribution systems in the United States. Following the initial biofilm incubation, 3×10^8 *L. pneumophila* cells were spiked into

each container. One mL water samples and 2 cm^2 biofilm samples were collected from each container periodically over 94 days. Prior to sampling, the water in each container was gently agitated to mix suspended *Legionella* while maintaining biofilm integrity. Biofilm samples were collected with sterile cotton swabs and resuspended in sterilized deionized water. CFU/mL and CFU/cm^2 were determined for each sample via spread plate technique, performed in duplicate for each sample, on BCYE agar medium.

2.3.5: Growth and survival of *Legionella* in a model water distribution system

The ability of introduced *Legionella* to colonize and survive within distribution system water and biofilms was established using a laboratory scale model water distribution system. The MDS had loop with PVC piping, consisting of a 5.5 m long and 5.1 cm in diameter main pipe, with a 1.4 m long dead-end, and a total volume of 50 liters. Distribution mains and dead-end lines consisted of 6 and 2 removable pipe sections (65 cm each in length), respectively. The main pipe was connected to a reservoir. A self-priming, thermally protected, magnetic-drive pump (Little Giant Pump Company, Oklahoma City, OK, USA) continuously re-circulated water between the main loop and the reservoir. Pressure, flow rate (0.304 m/s), and temperature (25 °C) were kept constant through external controls. The MDS had been used continuously for 11 years and contained well established biofilm communities before the start of this experiment.

City of Tempe, AZ, USA tap water was circulated in the MDS, following dechlorination. PVC coupons 2.5 cm in diameter and 5 cm in length were suspended in the reservoir for several weeks to allow biofilm formation. Prior to inoculation with a

laboratory strain of *L. pneumophila*, water and biofilm samples were tested to confirm the absence of culturable *Legionella* in the MDS. After inoculation with approximately 10^7 *L. pneumophila* cells, water and biofilm samples from the MDS were periodically collected over 131 days and cultured on BCYE to determine *Legionella* concentrations. Water samples were collected from two sampling ports: 1) one located in a dead end segment of the system approximately 3 m from the reservoir and 2) one located in an open segment of the system approximately 1.2 m from the reservoir. Two 5 mL water samples were collected from each port for each sampling event, one before and one after flushing one liter of water, with Legionella concentrations averaged between the two. This form of sampling was performed to assess any variation between *Legionella* in the samples due to potential water stagnation or biofilm for the ports. When necessary, water samples were concentrated via the membrane filtration technique using 0.45 micron pore size filters resuspened in 10 mL sterilized deionized water. Biofilm samples were collected from PVC coupons using sterile cotton swabs and resuspended in sterilized deionized water prior to spread plating. After 131 days, the MDS was treated with 5 mg/L of chlorine introduced into the reservoir. Two hours later, water samples were collected along with biofilm samples from a coupon, a dead end section, and an open end section of pipe.

2.3.6: Presence of *Legionella* in water meter biofilms

Residential drinking water meters were collected from two general areas located in central Arizona: 35 from area A and 32 from area B. Sampling occurred over a period of approximately one month during June/July 2011. Collection was possible due to water meter recycling programs in which water utilities replace mechanically worn meters with

new ones. All water meters collected were made of brass, with some containing rubber washers on their inlet rims. Upon removal by utility personnel, meters were submerged in tap water and transferred to the environmental microbiology laboratory at Arizona State University. Biofilm samples were collected from the inlet of each meter within two hours of removal. Approximately 40 cm^2 of biofilm were collected using sterilized nylon wire brushes and resuspended in 20 mL of sterile water. DNA extraction was performed on 750 µL of this suspension, followed by PCR amplification using specific primers for *Legionella* spp. and *L. pneumophila.* PCR reactions producing bands visible on a DNA gel were sequenced to identify the presence of *Legionella* or *L. pneumophila*. Biofilm samples positive for *Legionella* were cultured on BCYE via the spread plate technique.

2.3.7: Data analysis

Excel (Microsoft Corporaion, Redmond, WA, USA) was used for all data analysis and graph generation. Student's T-tests were performed, with a p-value cut-off value of 0.05 used to determine significance using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria),

2.4: Results and discussion

2.4.1: Survival of *Legionella* in tap water at 4, 25, and 32 °C

During the first week of incubation, no significant decrease in the concentration of *Legionella* was observed at any temperature (Fig. 1). At 4 °C, after the first week of a steady state, *Legionella* concentration decreased at an exponential rate for two months

before stabilizing at 10^2 CFU/mL for the remainder of the study period. At 25 °C, *Legionella* concentration decreased exponentially for 30 days and stabilized at approximately 10^5 CFU/mL. At 32 °C, *Legionella* concentration decreased at an exponential rate for 18 days and relatively stabilized at approximately 5×10^3 CFU/mL. After the initial decrease, the variation in concentration was more pronounced at 32 °C than for the other temperatures. After 11 months, *Legionella* concentrations at 4, 25, and 32 °C were <1 CFU/mL, <1 CFU/mL, and $>10^3$ CFU/mL, respectively, suggesting that long term survival of *Legionella* was supported by the higher temperature.

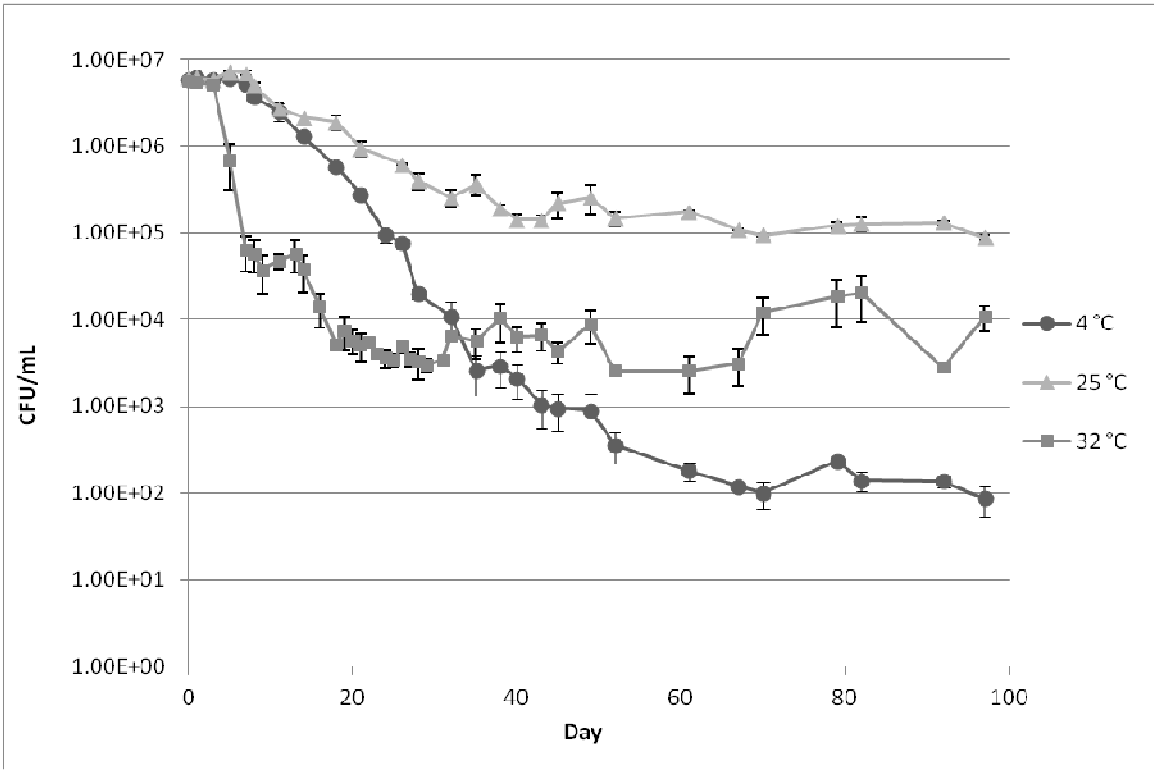

Fig. 1 - *L. pneumophila* concentration in 50 mL tap water incubated at 4, 25, and 32 °C over 97 days after inoculation with 3×10^7 cells. Day 0 represents sampling performed 2 hours post inoculation. Error bars indicate standard error between two replicate cultures.

The data from the initial three months of the experiment indicate a more stable population of *Legionella* at 25 °C compared to 4 °C and 32 °C. Conversely, culturable cells were only detected at 32 °C after 11 months. While periods of population maintenance were eventually established at each temperature, the cultures incubated at 25 °C reached a steady state quicker and with higher concentrations than at 4 or 32 °C. *Legionella* replication has been demonstrated to be faster with increasing temperature (Schulze-Röbbecke et al. 1987), raising the question as to why higher concentrations were observed in the initial three months of the experiment at 25 °C. The increased survival of the *Legionella* population observed at a lower temperature (25 °C versus 32 °C) during the initial three months of incubation may have been due to lowered metabolically linked cell turnover or similar factors. Cell cycling may have also contributed to the differences in concentrations observed at the two temperatures, as the highly pleomorphic *Legionella* spp. are known to alter cellular morphology in response to environmental stresses to aid in survival under extreme conditions (Al-Bana et al., 2013). High temperatures are known to induce formation of resilient (yet often non-culturable) filamentous *Legionella* (Piao et al., 2006), which may have factored into the increased long term survival observed at higher temperature. The rapid decrease in concentrations observed during the initial days of the experiment may have been linked to this phenomenon of the formation viable but non-culturable *Legionella*. This is particularly relevant in regards to filamentous cells, as a single multinucleate filament capable of giving rise to dozens of individual bacilli under specific environmental conditions may only produce a single colony when cultured. Regardless of cell cycling dynamics,

replication could have contributed to the increased survival observed at both higher temperatures compared to 4 °C. This appears to have been demonstrated by multiple concentration increase in *Legionella* population in the 32 °C incubation, including a one log increase between days 67 and 82. No such increase in population was recorded for the cultures incubated at the other temperatures. It should be noted that the long term survival and population stabilization from this experiment occurred in the absence of mature biofilm formation as the frequent agitation of the tubes prior to sampling should have minimized biofilm formation.

2.4.2: Association of *Legionella* with biofilms on a variety of pipe coupons

For all pipe materials, similar *Legionella* biofilm association (10^5 to 2.7×10^5 CFU/cm^2) was observed three days after inoculation (Fig. 2). After the initial 3 days, concentrations in biofilms formed on cast iron decreased before stabilizing at 1.2×10^3 CFU/cm^2 for the duration of the study, resulting in a 2-log reduction. Concentrations on copper remained stable near 10^5 CFU/cm^2 until day 50 and then decreased until day 90. Concentrations on brass increased 7-fold to a maximum of 7.6×10^5 CFU/cm^2 by day 14, and then steadily decreased until day 90. Concentrations on PVC followed a similar trend to brass, increasing to 1.6×10^5 CFU/cm^2 by day 21 before decreasing. Final concentrations on copper, brass, and PVC were similar at day 90, each near 10^4 CFU/cm^2, approximately a 1-log reduction from the initial spiked concentrations. *Legionella* levels in water surrounding each coupon (Fig. 3) eventually decreased, with final concentrations of 1.3×10^3, 2.1×10^3, 4.9×10^3, and 4.9×10^3 CFU/mL for brass, cast iron, copper, and

PVC, respectively. Concentrations were stable for 14, 8, 8 and 3 days in water surrounding the PVC, copper, brass, and cast iron coupons, respectively.

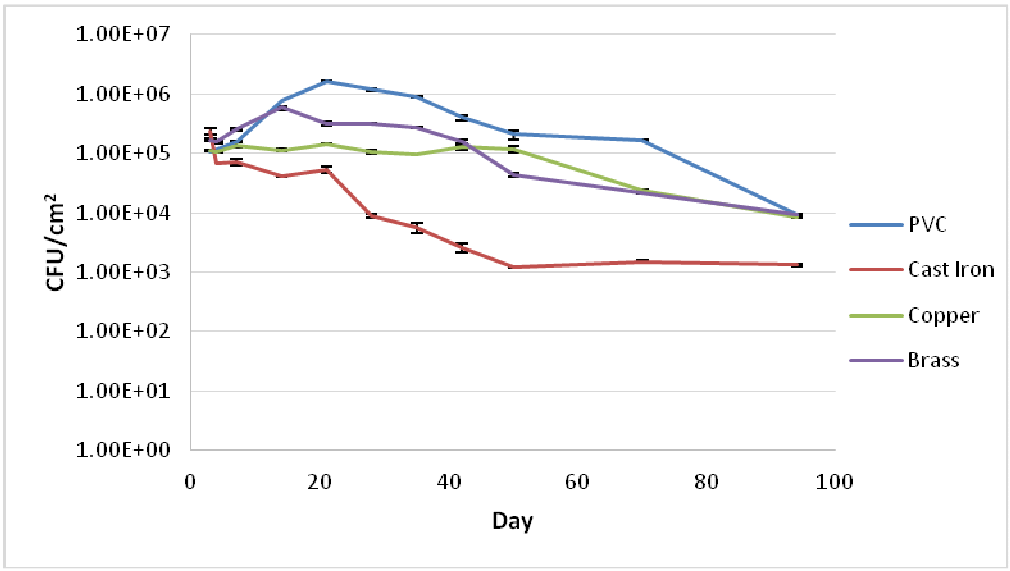

Fig. 2 - *L. pneumophila* concentrations in biofilms on the coupons submerged in tap water incubated at 25 °C. Error bars indicate standard error between duplicate samples of each culture.

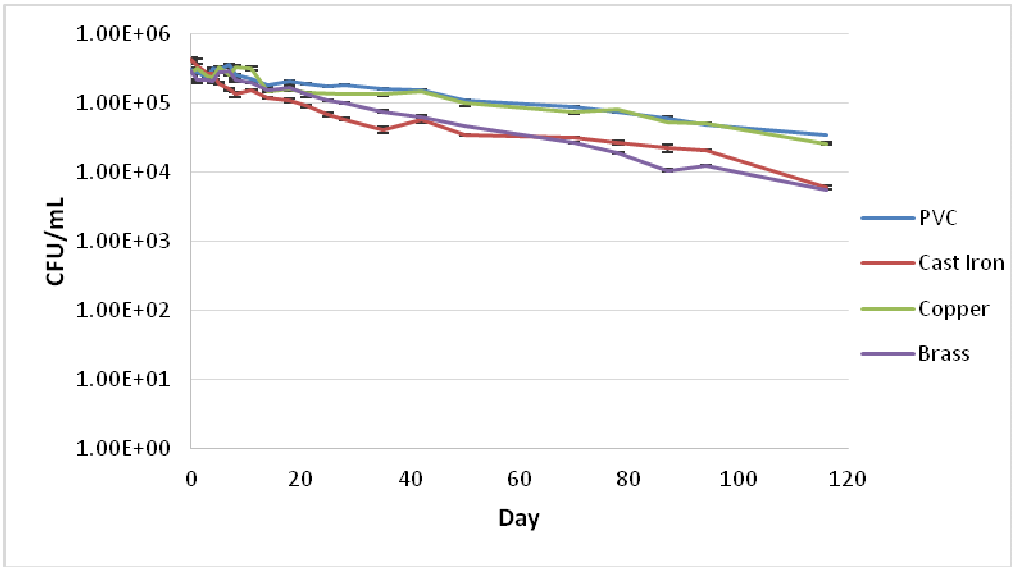

Fig. 3 - *L. pneumophila* concentrations in tap water containing the coupons incubated at 25 °C. Error bars indicate standard error between duplicate samples of each culture

The association of *Legionella* within biofilms on each coupon examined demonstrates the capability of this organism to establish a population on a wide variety of pipe materials. Two materials chosen due to their assumed antimicrobial properties, brass and copper (Pongratz et al., 1994), had consistently higher concentrations of *Legionella* in biofilms than on the cast iron coupon. While this may have been due to a combination of replication, different rates of colonization/release, or impacts on culturability within biofilms formed on cast iron, it appears *Legionella* may have increased affinity for these presumed antibacterial materials. Overall *Legionella* concentrations were at the highest on the PVC coupon, although the final recorded concentrations were similar for PVC, brass, and copper. These results suggest that, while pipe material may be a factor in the long-term survival of *Legionella* populations, the dynamics of *Legionella* persistence in distribution systems may rely more significantly on other factors, such as pipe age, water quality, and microbial community, which were unaccounted for in this batch study. The net concentration changes were similar between water and biofilms for each coupon, with the exception of cast iron, which demonstrated an additional 1-log reduction compared to the other coupons. Overall, steady reductions in *Legionella* concentration were measured for the water surrounding each material, while concentrations in biofilms experienced more drastic fluctuations. This discrepancy between concentrations over time in biofilms and water suggest dynamics such as biofilm

release/re-colonization, replication, or cell cycling (including transitions to viable but non-culturable states) may have been occurring, potentially most noticeably within biofilms on the cast iron coupon. As expected, *Legionella* population dynamics substantially varied amongst biofilms and water, characterized by periods of concentration increase, stability, and sharp decrease in biofilms, as opposed to steady decreases observed in water samples.

2.4.3: Growth and survival of *Legionella* in a model water distribution system

Within hours of inoculation, similar *Legionella* concentrations (approximately 1.5×10^3 CFU/mL) were detected in samples collected from both sampling ports (Fig. 4) of the MDS. During the first week, *Legionella* concentration rapidly fluctuated and then stabilized at approximately 8×10^2 CFU/mL. From day 20 through day 45, a steady increase in concentration was observed, with a peak concentration of 3.5×10^3 CFU/mL, followed by a steady decrease until day 60. After this point, *Legionella* concentration stabilized at approximately 1.6×10^3 CFU/mL until day 126. At day 131 a concentration of 4.3×10^3 CFU/mL was recorded, followed by 1.7×10^3 CFU/mL after chlorination. Three days after inoculation of the MDS, an initial biofilm concentration of 8.8×10^2 CFU/cm^2 was measured (Fig. 5). Concentration within biofilm samples increased steadily, with a peak at 2.2×10^4 CFU/cm^2 on day 28, followed by a steady decrease until reaching a stable concentration near 4.4×10^3 CFU/cm^2 on day 68 until day 131. After chlorination, biofilm concentrations (averaged from coupons and pipe loop segments) decreased to 1.1×10^2 CFU/mL. After 6 weeks, concentrations in both biofilms and the MDS flowing water increased to pre-chlorination levels (data not shown). In addition,

culturable *Legionella* were detected for over a year in the MDS, with approximately 1.5×10^3 CFU/mL in flowing water and 2×10^3 CFU/cm^2 in biofilm samples 13 months after the initial inoculation.

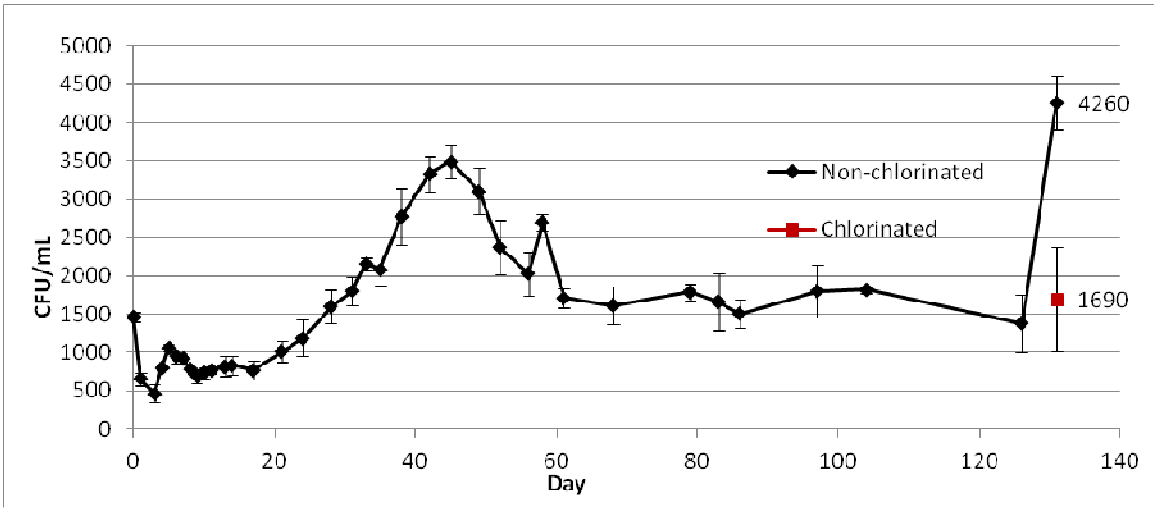

Fig. 4 - *L. pneumophila* in flowing water of a model drinking water distribution system over a period of 131 days after inoculation with 10^7 cells. Day 0 corresponds to sampling performed 2 hours after inoculation. The red square at day 131 corresponds to sampling performed 2 hours after chlorination. Error bars indicate standard error of samples collected from two separate ports before and after flushing 1 L of water.

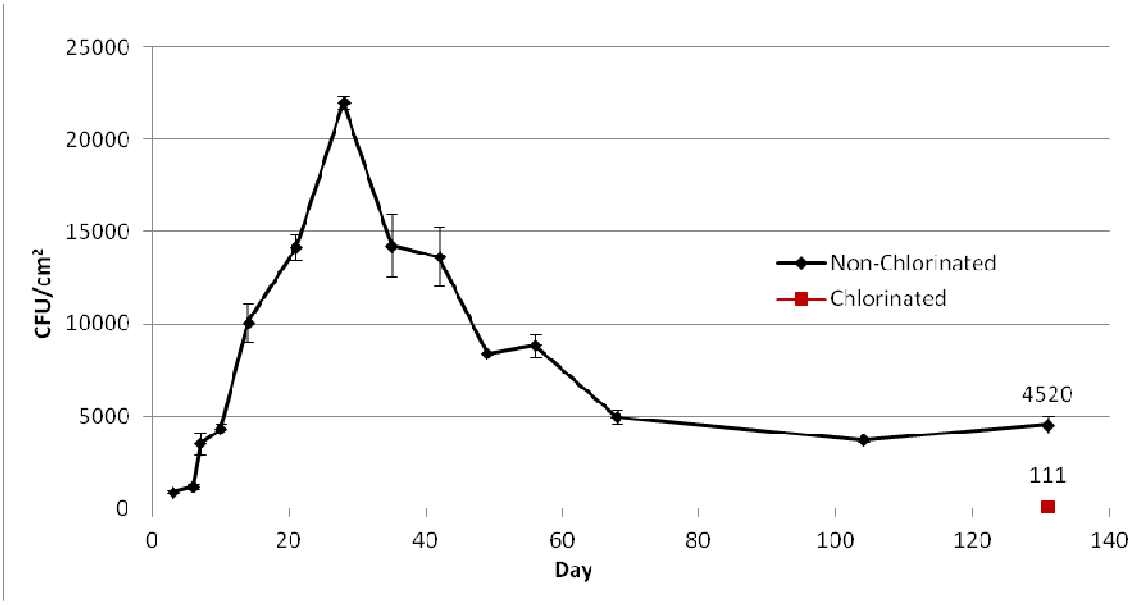

Fig. 5 - *L. pneumophila* in biofilms of a model drinking water distribution system over a period of 131 days after inoculation with 10^7 cells. The first data point corresponds to sampling performed 3 days post inoculation. The red square at day 131 corresponds to sampling performed 2 hours after chlorination. Error bars indicate standard error from two biofilm samples.

The initial fluctuation in the concentration of *Legionella* over the first 10 days after inoculation can be explained by dispersal throughout the MDS and colonization of biofilms. The spikes in concentration occurring at days 58 and 131 were potentially due to release of organisms from biofilms resulting from incidental agitation while removing pipe segments for biofilm sampling. This suggests analogous disruption events within a drinking water distribution system could cause increases in *Legionella* concentration in flowing water, resulting in an increased risk of exposure to *Legionella*. Because the vast majority of *Legionella* in the MDS were always biofilm associated (the system contained substantially more cm^2 of surface area than mL of flowing water), results also suggest

monitoring of only water samples may not provide an accurate estimation of the true level of contamination within a distribution system. Despite the two sampling ports used being located on opposite ends of the MDS with differing flow conditions, similar concentrations (1.44×10^3 CFU/mL and 1.48×10^3 CFU/mL) of *Legionella* were seen in the flowing water samples collected two hours after inoculation, suggesting a rapid and even dispersal throughout the MDS and within dead-ends. This was further supported by the fact that mean *Legionella* concentrations in samples collected from these two flow conditions remained relatively similar throughout the study. In addition, similar concentrations were observed in water samples collected before and after flushing the ports. It should be noted that the largest variation in water concentrations were measured in the samples collected after chlorination on day 131, possibly related to both differential dispersal of chlorine and sedimentation depending on flow conditions of pipe sections. After early fluctuations, *Legionella* concentrations in both flowing water and biofilms of the MDS reached stable levels for extended periods of time, with an initial decrease followed by stabilization. However, in biofilms the periods of increase and decrease occurred 14 days earlier than the flowing water samples. Chlorination resulted in significant reduction of *Legionella* within biofilms (1.4 log, p-value: 0.0419) and flowing water (0.5 log, p-value: 0.0314). The difference in the effectiveness of chlorination against *Legionella* present in water and biofilm seems contrary to the notion that biofilms typically provide additional protection from disinfection (Cooper et al. 2010). The reactive properties of the chlorine itself (alongside the previously mentioned physical disruption), may have played a role in increasing the concentration of *Legionella* in the flowing water in the system by inducing the release of *Legionella* containing biofilm

fragments into the flowing water. This demonstrates a potential scenario in which the concentration of *Legionella* or other biofilm dwelling pathogens could increase in the flowing water of a distribution system following disinfection. The eventual increase and stabilization observed for concentrations in both biofilms and flowing water following chlorination coincides with previous evidence highlighting the difficulty of treating distribution systems for *Legionella* (Marchesi, et al, 2011).

2.4.4: Presence of *Legionella* in water meter biofilms

Molecular analysis of the biofilms samples tested from the 35 water meters collected from Area A confirmed the presence of *Legionella* in 9 meters, with 5 samples also testing positive for *L. pneumophila* (Table 2). None of the 32 meters collected from system B tested positive. All attempts at culture confirmation for viable *Legionella* were unsuccessful due to high levels of non-legionella bacterial growth.

Table 2: *Legionella* and *L. pneumophila* occurrence via PCR in residential water meters biofilms.

Sample Site	*Legionella* spp. Positive	*L. pneumophila* Positive
System A	26% (9/35)	14% (5/35)
System B	0% (0/32)	0% (0/32)

The fact that biofilms collected from water meters tested positive for the presence of *Legionella* demonstrates a practical approach for future studies on the occurrence and prevalence of this pathogen in drinking water distribution system biofilms. While not perfectly representative of biofilms found elsewhere in a distribution network, those within the water meters present a source of samples widely accessible and numerous in any major metropolitan area. The possible effects of brass (as opposed to other distribution system pipe materials such as PVC and iron) on the survival and biofilm colonization of *Legionella* could have impacted the results of this study. These effects may be negligible, as results from this study have demonstrated the capability for *Legionella* to associate within biofilms formed on brass in a manner similar to other common pipe materials (Fig.2). More than half of the water meters biofilms positive for *Legionella* also tested positive for *L. pneumophila*, indicating that the species of *Legionella* most commonly implicated in human disease may also be relatively common in relation to other members of the genus in drinking water distribution systems. In addition, high occurrence in drinking water distribution systems across the United States (Donohue et al., 2014) suggests a widespread *Legionella* contamination may be typical. A number of explanations could be considered as to why Area B contained no water meters positive for *Legionella*, whereas those from Area A tested positive for both *Legionella* and *L. pneumophila*. While both Area A and Area B receive their water from the same three sources in central Arizona, the proportions from each source are not identical and varied during different seasons. In addition, Area B consists of a newer pipe network and water treatment methods (such as utilization of ozone) differed between the two. Further examination of these and other factors could determine how drinking

distribution systems may create environments that either inhibit or facilitate *Legionella* growth and survival.

2.5: Conclusions

- *Legionella* population maintenance is possible in unheated water systems
- *Legionella* biofilm association can occur on a variety of pipe materials in tap water.
- Water samples may not accurately reflect *Legionella* contamination conditions in distribution systems.
- *Legionella* contamination may vary drastically in different drinking water distribution systems.

With legionellosis cases continually on the rise in the United States and across the world, the development of appropriate public health protocols to curtail the incidence of this theoretically preventable disease is more important now than ever. Despite the fact that a substantial proportion of efforts focused on monitoring and treatment procedures aimed at preventing *Legionella* contamination in-premise plumbing, the dispersal and contamination of *Legionella* in these systems is clearly an important factor to consider. The very water distribution systems that introduce these pathogens to building plumbing systems are a logical target to help prevent legionellosis incidence. By examining *Legionella* survival in tap water, association in pipe material biofilms, interactions in a model distribution system, and prevalence in drinking water distribution system biofilms, the results of this study provides valuable information relevant to the design of

monitoring and control procedures for *Legionella* in public drinking water.

CHAPTER 3

NUTRIENT PRESENCE AND ACQUISITION FOR *LEGIONELLA*

3.1: Abstract

Legionella are a common yet poorly understood water-born pathogen. These bacteria are ubiquitous in water systems, where protozoan host endoparasitization and biofilm association play important roles in their life cycle. While commonly thought to obtain the majority of their nutrients from host organisms, *Legionella* are capable of deriving nutrients necessary for replication from other sources, including via necrotrophy. To determine the presence and source of two essential nutrients for *Legionella* growth, iron and L-cysteine, environmental water samples were collected and separated via filtration into different components, with freeze-thawing used to release potential nutrients from microbial cells in the samples. These components were supplemented into modified BCYE media prepared with the following conditions: 1) no iron or L-cysteine added, 2) iron added, 3) L-cysteine added, and 4) both iron and L-cysteine added. Growth of *L. pneumophila* on these media demonstrated levels of iron, L-cysteine, or both, in sufficient concentrations to support *Legionella* growth in environmental waters outside of host cells. Another experiment measured *L. pneumophila* growth in the previously mentioned components compared with laboratory grade sterile water. Results showing higher concentrations of *Legionella* in comparison to sterile water suggest that certain components in environmental water are capable of supporting the growth of this pathogen. The results from this study serve to further elucidate the environmental nutritional requirements for *Legionella* and potentially demonstrate their capability for environmental growth in the absence of host organisms.

3.2: Introduction

Since the discovery of Legionnaires' disease in 1976, bacteria of the *Legionella* genus (Brenner et al., 1979), particularly *L. pneumophila* have become water-borne pathogens of significant concern. While great strides in *Legionella* research have been accomplished since the discovery of the bacteria, steadily increasing incidence of outbreaks caused by *Legionella* (Brunkard et al., 2011, Hicks et al., 2011) are most certainly due, in part, to a lack of understanding of key aspects of the organisms physiology and ecology. Such paucity exists for crucially important characteristics of *Legionella*, such as the effects of environmental conditions on survival, interactions and growth dynamics in water distribution systems, and nutritional requirements for environmental growth. *Legionella* are considered to be fastidious organisms, requiring high concentrations of iron and L-cysteine for growth on culture media. While these nutrients are typically extracted from eukaryotic host organisms such as amoeba in the environment, necrotrophic growth of *Legionella* on dead bacterial cells has been documented *in vitro* (Temmerman et al., 2006), demonstrating the potential for growth outside of a host cell. These examples highlight relevant areas of *Legionella* research which this study aims to elucidate through a series of bench and pilot scale experiments.

3.3: Methods

3.3.1: Microbiological methods

All *Legionella* culturing techniques used in this study were derived from those laid out by the United States Centers for Disease Control and Prevention (CDC 2005). Samples were cultured on buffered charcoal yeast extract media (BCYE Agar Base, Benson, Dickson, and Company, Franklin Lakes, NJ, USA) with the following supplements: 0.3% glycine, 100 units/mL polymixin B, 5 µg/mL vancomycin, and 80 µg/mL cyclohexamide. *L. pnuemophila* stock cultures were grown at 37° C with agitation over 72 hours in charcoal yeast extract broth media. Cell concentration in stock cultures was estimated using measurements of optical density at 600nm. All cultures were incubated at 37° C for 72 hours with up to 7 additional days as needed. All laboratory experiments utilized the *L. pneumophila* serogroup 1 strain Knoxville-1 (American Type Culture Collection 33153).

3.3.2: Environmental water sampling

For experiments testing nutrient requirements for *Legionella*, four sets of water samples were used: 1) water from a home aquarium, 2) environmental water originating from a ground water distribution system, 3) primary treated wastewater, and 4) reclaimed wastewater. Sample sites were chosen based on the likelihood of each water type being able to support *Legionella* growth via dissolved nutrients and/or potential *Legionella* host organisms. In addition, water collected from the second site had previously demonstrated

high levels of *Legionella* growth (unpublished data). Immediately upon sampling, water samples were stored at 4° C until processed.

3.3.3: Separation of environmental water components

The previously mentioned environmental water samples were separated into four separate components using varying pore size filter discs: 1) whole unfiltered sampled, 2) elution of resuspended cells trapped in a 1.2 µm filter, 3) filtrate of water and dissolved chemicals passed through a 0.2 µm filter, and 4) filtrate of water and dissolved chemicals passed through a 0.2 µm filter after lysis of microbial cells in the original sample. The 1.2 µm elutions were resuspended in sterile water via vortexing. Lysis was performed via a freeze-thawing process involving a 30 minute incubation at -80° C followed immediately by a 30 minute incubation at 45° C.

3.3.4: Qualitative growth of *Legionella* on media supplemented with nutrients from environmental water

To qualitatively measure the growth of *Legionella* in environmental waters, a microbiological assay was designed revolving around the use of modified BCYE media supplemented with the previously mentioned components of primary treated wastewater and reclaimed water. Four forms of modified BCYE media were prepared: 1) containing no iron or L-cysteine, 2) containing iron but not L-cysteine, 3) containing L-cysteine but no iron, and 4) containing iron and L-cysteine. While preparing the media, 50% of the typical sterile water content was replaced with either filtrate or lysis filtrate from

wastewater or reclaimed water, resulting in a total of 16 media types containing environmental water components. In addition, a set of four control media types was prepared containing 100% of the typical sterile water content as standard BCYE and no environmental water components. The four control media types were poured with 150% the volume of the test media types. After solidifying, each media type was spotted with separate suspensions of *L. pneumophila* comprised of three strains: 1) the laboratory strain Knoxville-1, 2) an environmental strain isolated from tap water in Central AZ, USA (environmental strain 1), and 3) an environmental strain isolated from ground water in Central AZ, USA (environmental strain 2). The plates were incubated for a total of 14 days, and the presence and quality of growth was recorded.

3.3.5: Quantitative growth and survival of *Legionella* in environmental waters

To quantitatively measure the growth of *Legionella* in environmental waters, cultures of the *L. pneumophila* strain Knoxville-1 were inoculated into 2.5 mL of various components of the several environmental waters and incubated at 37° C with agitation, with CFU/mL measured over time. Three separate experiments were conducted. In the first test, aquarium water elution, and aquarium water lysis filtrate (both from environmental water sample 1) were compared. In the second test, the following components from environmental water originating from ground water (environmental water sample 2) were compared: whole sample, elution, filtrate, and lysis filtrate. In the third test, whole sample and elution from wastewater and reclaimed water (environmental water samples 3 and 4) were compared. Each test also contained a culture of *Legionella* in sterile water as a control.

3.4: Results and discussion

3.4.1: Growth of *Legionella* on media supplemented with nutrients from environmental water

Data from the qualitative growth tests of *Legionella* via nutrients from environmental waters are depicted in tables 3, 4, and 5. All strains showed strong growth in all media supplemented with iron and L-cysteine. Strong growth was also seen for the lab strain and environmental strain 2 on the control media containing L-cysteine. No growth was seen for the lab strain on any other media. No growth was seen for environmental strain 1 on control media not supplemented with both iron and L-cysteine. Microcolony formation was observed on several test media types, including weak true colony formation on reclaimed water filtrate media supplemented with L-cysteine and wastewater lysis filtrate media supplemented with L-cysteine. No growth was seen for environmental strain 2 on control media without supplemented L-cysteine and microcolony formation was observed on several test media types. For all strains, growth observed on wastewater lysis filtrate media was faster and more robust than on wastewater filtrate media. Growth was similar on reclaimed water lysis filtrate and reclaimed water filtrate for all strains. For the lab strain and environmental strain 2, growth on control media supplemented with only L-cysteine was slower, less robust, and resulted in atypically colored colonies (pale green as opposed to pale blue).

Table 3: Growth of *L. pneumophila* ATCC strain 33153 on modified BCYE supplemented with components of environmental water samples.

Lab Strain	-Fe/-Lc	+Fe/-Lc	-Fe/+Lc	+Fe/+Lc
Control	-	-	C	C
Reclaimed Filtrate	-	-	-	C
Reclaimed Lysis Filtrate	-	-	-	C
Wastewater Filtrate	-	-	-	C
Wastewater Lysis Filtrate	-	-	-	C

Water sample component used is listed in column one. Iron/L-cysteine content is listed in row one. C: robust colony formation. -: no colony formation.

Table 4: Growth of *L. pneumophila* environmental strain 1 on modified BCYE supplemented with components of environmental water samples.

Environmental Strain 1	-Fe/-Lc	+Fe/-Lc	-Fe/+Lc	+Fe/+Lc
Control	-	-	-	C
Reclaimed Filtrate	M	M	C*	C
Reclaimed Lysis Filtrate	-	M	M	C
Wastewater Filtrate	-	-	-	C
Wastewater Lysis Filtrate	M	M	C*	C

Water sample component used is listed in column one. Iron/L-cysteine content is listed in row one. C: robust colony formation. C*: weak colony formation. M: microcolony formation. -: no colony formation.

Table 5: Growth of *L. pneumophila* environmental strain 2 on modified BCYE supplemented with components of environmental water samples.

Environmental Strain 2	**-Fe/-Lc**	**+Fe/-Lc**	**-Fe/+Lc**	**+Fe/+Lc**
Control	-	-	C	C
Reclaimed Filtrate	M	-	M	C
Reclaimed Lysis Filtrate	M	M	M	C
Wastewater Filtrate	M	M	-	C
Wastewater Lysis Filtrate	M	M	M	C

Water sample component used is listed in column one. Iron/L-cysteine content is listed in row one. C: robust colony formation. C*: weak colony formation. M: microcolony formation. -: no colony formation.

The increases in growth demonstrated by all strains on wastewater lysis filtrate media compared to wastewater filtrate media suggest that *Legionella* were able to utilize nutrients originating from lysed microbial cells. The fact that this phenomenon was not observed in reclaimed water media provides further support for this, as the wastewater used in the media presumably contained several orders more microbes than the reclaimed water used. Theoretically, if an environmental water source contained high levels of microbial cells exposed to some form of pressure resulting in large-scale cell death while simultaneously having no effect on *Legionella* in the water, the result could be a supply of nutrients to support *Legionella* growth outside of a host cell. The growth of environmental *Legionella* on wastewater and reclaimed water filtrate media also suggests that these waters may contain dissolved iron and/or L-cysteine in quantities sufficiently high enough to support growth. The increased robustness of growth on media supplemented with L-cysteine as opposed to iron (including cases of true colony as opposed to microcolony formation for environmental strain 1) suggests that these two water types may contain relatively higher levels of readily available iron than L-cysteine.

The growth of the lab strain and environmental strain 2 on control media supplemented with L-cysteine is interesting, as levels of iron in base BCYE media are typically insufficient to support *Legionella* growth. The weak growth accompanied by an atypical colony morphology suggests that bacteria were struggling to grow. *Legionella* are thought to require somewhere between 1-10 µM iron at the absolute minimum to culture on media (Reeves et al., 1981), while BCYE typically contains nearly 1400 µM.

A component of BCYE, yeast extract, contains trace amounts of iron, and by increasing the volume of media poured into the control media plates in this experiment, iron levels appear to have been elevated high enough to support growth of two of the strains tested. The fact that environmental strain 1 showed no growth (even the formation of microcolonies was not observed) in the same nutrient conditions suggests that nutritional requirements can vary significantly amongst strains of *L. pneumophila*. This notion is further supported by the lack of growth of the lab strain on any test media not supplemented with both iron and L-cysteine, and the differing growth patterns on tests media types for the two environmental strains.

3.4.2: Quantitative Growth and Survival of *Legionella* in Environmental Waters

Data for the survival of *Legionella* in aquarium water components, aquarium water elution, and sterile water (Fig. 6) show a relatively steady near exponential decrease in *Legionella* concentrations throughout the entire period of the test. Conversely, concentrations from the aquarium water lysis filtrate show a decrease after 5 days to 2.6×10^5 CFU/mL, followed by an increase to 7.2×10^5 CFU/mL, before steadily decreasing to a final concentration of 6×10^4 CFU/mL at day 46. Data from for survival in components of environmental water sample 2 (Fig. 7) show a steady exponential decrease in *Legionella* concentrations for sterile water, along with varying rates and ranges of decrease and concentration maintenance for environmental sample two whole sample, elution, and lysis filtrate, with final concentrations for the three components at day 33 were: 4×10^4 CFU/mL, 2×10^3 CFU/mL, and 1.2×10^2 CFU/mL, respectively. Concentrations in environmental water sample 2 filtrate sharply decreased before

reaching undetectable levels at day 8. Data for survival in components of wastewater and reclaimed water (Fig. 8) show a small delay before steady exponential decrease in concentration for *Legionella* incubated in sterile water and reclaimed water elution. A sharp, immediate loss of concentration was observed for the whole wastewater culture. Relatively high survival, including periods of concentration increase were observed for whole reclaimed water sample and wastewater elution.

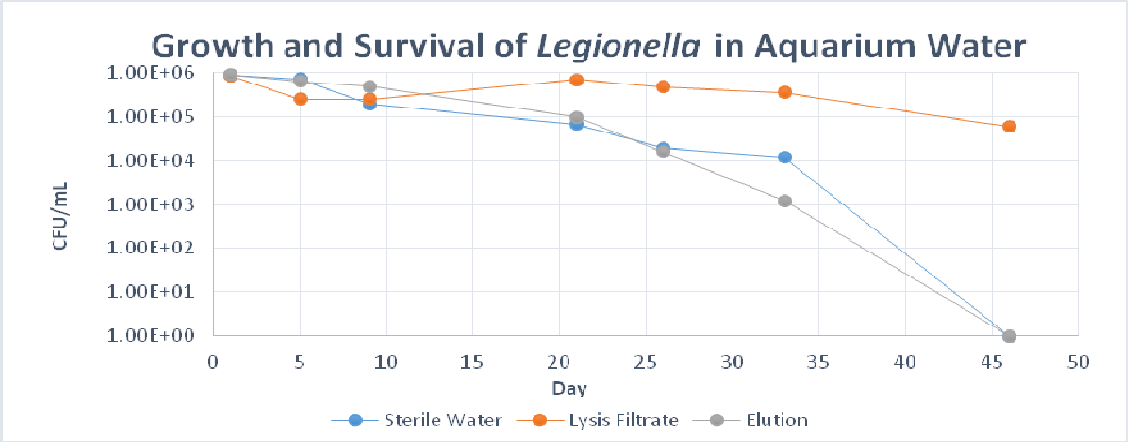

Fig. 6 - CFU/mL of *L. pneumophila* in 2.5 mL aquarium water elution and lysis filtrate over a period of 46 days at 37 °C after inoculation with 10^6 cells.

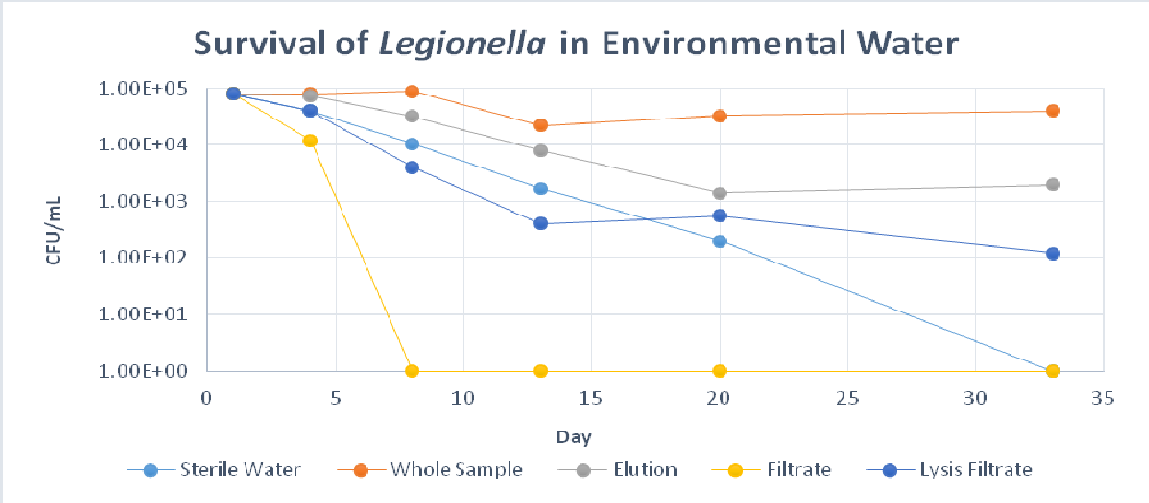

Fig. 7 - CFU/mL of *L. pneumophila* in 2.5 mL environmental water sample 2 whole sample, elution, filtrate, and lysis filtrate over a period of 33 days at 37 °C after inoculation with 10^5 cells.

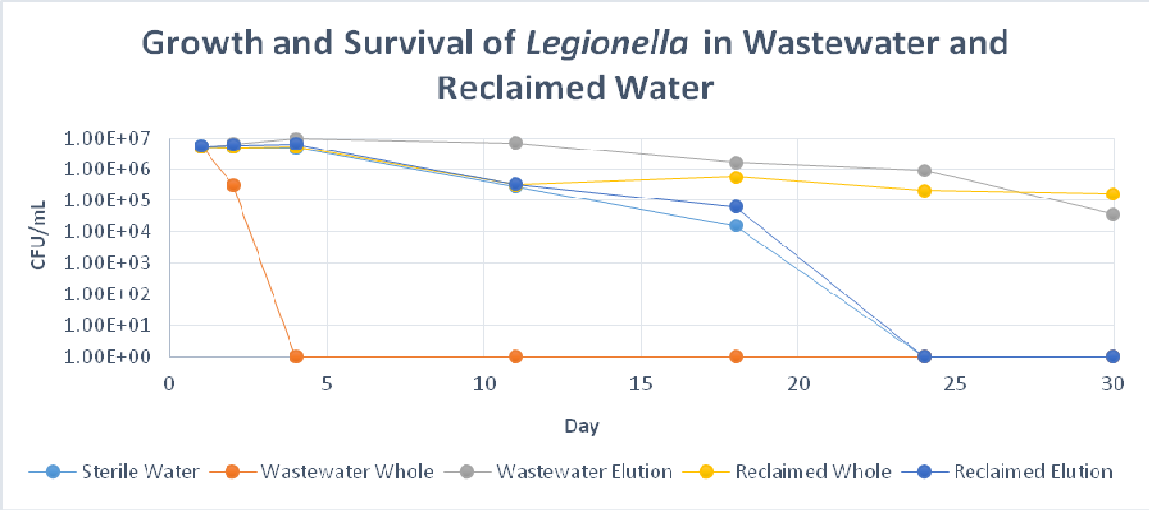

Fig. 8 - CFU/mL of *L. pneumophila* in 2.5 mL wastewater and reclaimed water whole sample and elutions over a period of 33 days at 37 °C after inoculation with 5.5×10^6 cells.

Results from these experiments demonstrate growth and/or increased survival of *Legionella* in a variety of environmental waters, both in and out of host cells. The nearly identical survival rates for *Legionella* cultured in sterile water and aquarium water elution (Fig. 6) suggest a lack of sufficient host cells for population growth. High survival rates, including concentration increases observed in aquarium water filtrate containing lysed cell components, indicates the potential for necrotrophic growth in this water. The increase survival rates in lysed filtrate compared to filtrate, along with significantly increased survival for *Legionella* cultured in whole sample compared to elution components indicate that *Legionella* may be acquiring nutrients from a non-host source in environmental water sample 2 (Fig. 7). Similarly to the aquarium samples, the increased survival and occasional growth observed in whole reclaimed water compared to reclaimed water elution depicted in Figure 8 indicate possible extra-host cell growth of *Legionella*. Incidentally, the nearly complete loss of *Legionella* in whole wastewater indicate the presence of growth inhibition in this water sample, while the high survival rates and growth observed for the wastewater elution culture demonstrate the presence of potential host organisms. By demonstrating increased survival and population increases for *Legionella* cultured in these various components of environmental water samples, growth dynamics of this pathogen have been examined further. In addition, the evidence suggesting growth of *Legionella* outside of a host cell further support claims generated from the qualitative growth experiments in this study.

3.5: Conclusions

- *Legionella* are capable of obtaining nutrients for growth from non-host sources.

- Amoebae are not necessary for long-term persistence of *Legionella*.
- Microbial communities can have a drastic impact on *Legionella* growth and survival.
- Necrotrophic growth can lead to *Legionella* population increase.
- Varying strains of *Legionella* demonstrate differing nutritional requirements.

Legionella continues to be a drinking water pathogen of increasing concern. As incidence of legionellosis rise each year, it becomes apparent that appropriate maintenance and monitoring protocols are needed to effectively disease caused by these bacteria. Before these protocols can be developed, further knowledge on their ecology needs to be elucidated to help us gain a sufficient understanding of how *Legionella* interact with their environment. By demonstrating the effect of temperature on growth, examining interactions within distribution systems, and investigating nutritional requirements for *Legionella*, these existing knowledge gaps will hopefully be reduced. The evidence produced through this study suggesting the potential for *Legionella* growth outside of a host organism is of particular relevance, and could have serious implications for future *Legionella* treatment procedures.

CHAPTER 4

AUTOMOBILE WINDSHIELD WASHER FLUID AS A POTENTIAL SOURCE OF *LEGIONELLA* TRANSMISSION

4.1: Abstract

Infections caused by *Legionella* have been traced to a wide variety of sources, and epidemiological evidence suggesting that driving cars is a risk factor for legionellosis has prompted public health studies to investigate vehicle windshield washer fluid as a novel transmission source. To date, a single study conducted in the United Kingdom has attempted to investigate the presence and survival of *Legionella* in automobile windshield washer fluid, with mixed results. The goal of this study was to investigate whether windshield washer fluid could serve as a potential source of transmission for *Legionella*. A wide variation in the survival of *L. pneumophila* was observed when incubated in different types of washer fluids at 25 and 37 °C, however, one brand of windshield washer fluid demonstrated *Legionella* survival potential similar to sterilized deionized water. In addition, one liter of tap water contained in a washer fluid reservoir was able to support population growth and survival of *Legionella* for several months. In a field study examining the windshield washer fluid of 12 elementary school buses, *Legionella* were detected from 84% of samples at a high concentration of 8.1×10^4 CFU/mL. Culturable cells were also detected in aerosolized washer fluid during washer fluid spray. By demonstrating survival in certain windshield washer fluids, growth within washer fluid reservoirs, and the presence of viable cells in bus washer fluid spray, we have provided evidence suggesting the potential for a novel route of *Legionella* exposure. The warm

climate of central Arizona, USA, in which the study was conducted, may have largely contributed to the results observed.

4.2: Introduction

Legionella species, particularly *L. pneumophila*, are responsible for more drinking water and non-recreational water-borne disease outbreaks in the United States than any other microbe (Brunkard et al., 2011). Incidence of legionellosis has consistently and significantly risen (Hicks et al., 2011) since the discovery of the disease in 1976 (Fraser et al., 1977). The majority of cases of legionellosis are caused by exposure through the respiratory route, however, atypical forms of the disease have been reported (Kilborn et al., 1992, McCabe et al., 1984, Yu 1993). The two most common forms of illness caused by this pathogen are: Pontiac Fever, resulting in flu like symptoms, and Legionnaires' disease, a potentially deadly pneumonia (Cordes and Fraser 1980).

Public health risk from *Legionella* is often associated with environmental conditions conducive to the growth of the organism (Storey et al., 2004), such as biofilm development, protozoan activity, and high temperatures (Borella et al., 2005). Reported outbreaks of legionellosis are commonly traced to sources with potential for aerosolization of high temperature water, such as cooling towers and spas (Fields et al., 2002), though unheated water systems such as decorative fountains and tap water distribution systems have also been linked to transmission (Hlady et al., 1993). Although well documented routes of exposure may be responsible for the majority of legionellosis outbreaks, sources of transmission are not always identified and unusual or poorly

understood reservoirs for these pathogens do exist (Arvand and Hack 2013, Sakamoto et al., 2009).

Epidemiological studies have suggested automobiles to be a possible source of transmission for *Legionella.* Research performed in the Netherlands (Den Boer et al, 2006) and Turkey (Polat et al., 2007) has shown an increased risk for Legionnaire's disease in professional drivers, while a study conducted in the United Kingdom (Wallensten et al., 2010) determined a novel risk factor for legionellosis to be driving in a car containing water in place of washer fluid. In addition, studies performed in Japan (Sakamoto et al, 2009), Greece (Alexandropouou et al., 2013), and the United Kingdom (Palmer et al., 2012) have detected *Legionella* in car air conditioning systems, car cabin air filters, and windshield washer fluid reservoirs without added washer fluid, respectively. In response to mounting evidence produced by these and similar studies, a series of survival experiments and a corresponding field study were conducted with the goal of assessing the potential for *Legionella* exposure from automobile windshield washer fluid.

4.3: Methods

4.3.1: Media and laboratory strain of *Legionella*

All laboratory experiments were performed using a stock of *Legionella pneumophila* ATCC strain 33152 (American Type Culture Collection, Manassas, VA, USA) cultured in Buffered Yeast Extract (BYE) medium. Laboratory and environmental water samples were assayed for the detection of *Legionella* using Buffered Charcoal

Yeast Extract agar (BCYE) medium (Procedures for the recovery of *Legionella* from the environment, 2005). BYE medium contained: 10.0g yeast extract, 0.25g ferric pyrophosphate, and 0.4g L-cysteine HCl per liter of distilled water. BCYE medium consisted of BD BBL Buffered Charcoal Yeast Agar (Diagnostic Systems, Sparks, MD, USA) supplemented with 0.4 g/1000 mL L-cysteine HCl, 0.3% glycine, 100 units/mL polymixin B, 5µg/mL vancomycin, and 80 µg/mL cycloheximide. Bacterial stocks were prepared by culturing *Legionella* in BYE medium in a tabletop shaker incubator at 37 °C under atmospheric CO_2 for 72 hours before quantification via optical density and enumeration of colony forming units (CFU) via the spread plate technique. Stock cultures were washed via centrifugation and resuspension in sterile deionized water to remove medium prior to incoculation.

4.3.2: Environmental sampling and culturing

Environmental water and air samples were collected using previously described methods (Procedures for the recovery of *Legionella* from the environment, 2005). *Legionella* concentrations were determined for all liquid samples via the spread plate technique. Plates were incubated at 37 °C under atmospheric CO_2 between 3 and 7 days and colonies were recorded. Prior to spread plate assays, certain environmental water samples were subjected to a heat treatment at 50 °C for 30 minutes to reduce the growth of non-*Legionella* organisms (Wullings et al., 2011).

4.3.3: Survival of *Legionella* in windshield washer fluid

Laboratory experiments were conducted using three brands (labeled A, B, or C) of windshield washer fluid prepared, following the manufacturers' suggested procedures, with sterilized deionized water. The components of each fluid according to manufacturers are listed in Table 1. To determine the survival of *Legionella* in the windshield washer fluids, a series of 50 ml polyethylene (PE) tubes were initially filled with fluids A, B, or C at half the manufacturer recommended concentration, or sterilized DI water. The windshield washer fluids were diluted in this initial experiment to maintain washer fluid concentrations feasibly occurring in actively used automobiles. Each PE tube was spiked with 1.5×10^6 CFU/mL of *L. pneumophila* and incubated at 37 °C. This temperature was chosen to simulate the high temperatures potentially reached in washer fluid reservoirs. Duplicate samples of each fluid were initially analyzed after 24 and 48 hours of incubation to determine *Legionella* concentrations via culturing. Thereafter, samples were periodically collected and analyzed up to 73 days or until a concentration of < 1 CFU/mL was reached.

A second set of 50 ml PE tubes filled with fluid A, fluid B, sterilized DI water, 10% methanol, or 20% methanol was prepared. Each tube was spiked with 1.5×10^6 CFU/mL of *L. pneumophila* and incubated at 25 °C, a temperature previously demonstrated to support long-term survival of *Legionella* in water (Schwake et al., 2012). Duplicate samples were periodically collected and *Legionella* concentrations in all PE tubes were periodically measured via culturing for up to 70 days or until a concentration of <1 CFU/mL was reached.

4.3.4: Growth of *Legionella* in windshield washer fluid reservoirs

To measure the growth of *Legionella* in windshield washer fluid reservoirs under laboratory conditions, two 1 L PE reservoirs (Interdynamics, Inc., Tarrytown, NY) were filled with 750 mL of dechlorinated municipal tap water (Tempe, AZ, USA) with no initial detectable *Legionella* colonies in 1 mL. An additional two reservoirs were filled with tap water from a laboratory model drinking water distribution system previously spiked with *L. pneumophila* containing approximately 2.5×10^3 CFU/mL of *Legionella*. Each reservoir was incubated at 25 °C or 37 °C. Samples were collected in duplicate and cultured periodically over the course of 75 days to determine *Legionella* growth. Prior to each sample collection, the water in each reservoir was mixed by gentle pipetting to minimize the disruption of potential biofilm formation. The fluid in the reservoirs was sampled to measure suspended cells potentially able to be aerosolized during windshield washer fluid spray. Biofilms were intentionally undisturbed to support potential biofilm associated growth of *Legionella* in the reservoirs.

4.3.5: Environmental detection of *Legionella* in school bus windshield washer fluid reservoirs

A field study was conducted for the detection, quantification, and identification of *Legionella* in windshield washer fluid from school buses. Three sets of windshield washer fluid samples were collected from a fleet of buses considered actively in operation and belonging to a school district in central Arizona, USA. Samples were collected from the washer fluid reservoirs of buses parked in the school district's maintenance yard on May 30, July 2, and July 31, 2012 between 11 am and 4 pm.

Sample volumes ranging from 50 to 250 mL were collected from a total of 12 school buses. In addition, samples were collected from the stock solution used to prepare the windshield washer fluid for the buses, tap water from a drinking water fountain in the maintenance yard garage, and a sink faucet in the maintenance yard garage. The stock solution for the bus windshield washer fluid reservoirs was reportedly prepared in batches of approximately 10 L at a time (based on demand) and stored in a sealed container in an air conditioned garage. Prior to collecting tap water samples, approximately 1 L of water was flushed from the drinking water fountain and the sink faucet.

Over the course of the study, additional buses were sampled and additional forms of relevant data were collected. On the initial sampling event, four buses, the washer fluid stock solution, and the sink faucet were sampled. On the second and third sampling events, an additional eight buses and the drinking fountain were also sampled. A single bus was unavailable for sampling on the second sampling event and the washer fluid stock solution was unavailable for sampling on the third sampling event. Air samples were collected from selected buses on the second and third sampling events. Air samples were taken using a PBI SAS-Super ISO Air Sampler (VWR International PBI S.r.L, Vio San Giusto, Milano, Italy) directly onto BCYE medium. For each sample, 0.5 m^3 of air was collected by holding the air sampler 1.5 m high while standing 1 m in front of a bus spraying several bursts of washer fluid in 30 second intervals. Archived weather data were accessed to collect information for the local daily high air temperature during each sampling event and the temperature of each liquid sample was recorded on the third sampling event. *Legionella* concentrations were determined for liquid samples via the spread plate technique. Selected liquid samples and isolated colonies cultured from field

samples were assayed by PCR using *Legionella* spp. and *L. pneumophila* specific primers followed by sequencing for confirmation.

Windshield washer fluid reservoir volume and engine placement for each bus was recorded, along with the operational status of each vehicle in the week that the sampling was performed (data not shown). Windshield washer fluid reservoirs in the front engine buses were located in the engine compartment within 0.5 m of the engine block, while reservoirs in rear engine buses were located several meters away from the engine block. The bus windshield washer fluid reservoirs sampled were reportedly filled with one of the two following washer fluids (Table 6); fluid D contained an unknown mix of proprietary cleaning agents, while fluid E contained 0.67-1% ethanol and 0.33% tetrasodium ethylenediamine tetraacetate. Both of these washer fluids were reportedly prepared from a concentrate with tap water from the sink faucet.

4.3.6: DNA extraction and molecular analysis

DNA extraction was performed on washer fluid samples and isolated colonies using a ZYMO Research yeast/bacterial DNA extraction kit (Zymo Research Corporation, Irvine, CA, USA). The primers used in the study are listed in Table 7. For both primer sets, PCR amplification mixtures consisted of: 12.5 µL Promega GoTaq Green MasterMix (Promega Biosciences LLC., San Luis Obispo, CA, USA), 10 µL DNA template, and 0.13 µM each primer, with a final reaction volume of 25 µL. Gel electrophoresis was performed in a 40 mL 1% agarose gel containing 2µL of 10,000X Invitrogen SYBR Safe DNA Gel Stain (Life Technologies Corporation, Carlsbad, CA, USA) to detect PCR products. In addition, sequencing was performed to determine the

species of *Legionella* in samples. The National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) (National Library of Medicine, Bethesda, MD, USA) was used to compare the amplified sequences to the NCBI database. 16S rRNA gene sequence homology greater than 98% was used as the criterion for speciation.

4.3.7: Data analysis

Excel (Microsoft Corporation, Redmond, WA, USA) was used for all data analysis and graph generation. The $\log_{10}$ reduction times (T_{90}) for *Legionella* in the different fluids examined were calculated via regression analysis. A polynomial regression line was fit to the observed data and the T_{90} value in days was calculated by solving for a 90% reduction from the concentration at day 0. Student's T-tests were performed, with a p-value cut-off value of 0.05 used to determine significance using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Table 6: Fluids used in *Legionella* survival and field studies

Fluid	Ingredients
Washer Fluid A	~1% methanol and unknown mix of cleaning agents
Washer Fluid B	0.03-0.16% 2-butoxy ethanol, methanol, and isopropanol with a combined percentage no higher than 0.18% and an unknown mix of cleaning agents
Washer Fluid C	0.25-1.25% isopropanol, 0.025-0.25% ethylene glycol, and an unknown mix of 4 proprietary cleaning agents
Washer Fluid D	Unknown mix of proprietary cleaning agents, some hazardous
Washer Fluid E	0.67-1% ethanol and 0.33% tetrasodium ethylenediamine tetraacetate
Methanol	10% methanol and 20% methanol diluted in sterilized DI water
DI water	Sterilized deionized water
Tap Water	Municipal tap water collected from a laboratory faucet

Table 7: List of primers used for PCR and sequencing

Primers	Sequences (5'→3')	Gene Amplified	Approximate Amplicon Length	Reference
LEG-226 LEG-858	AAGATTAGCCTGCGTCCGAT GTCAACTTATCGCGTTTGCT	*Legionella* 16s rRNA	654 bp	18
LpneuF LpneuR	CCGATGCCACATCATTAGC CCAATTGAGCGCCACTCATAG	*L. pneumophila mip*	150 bP	18

4.4: Results

4.4.1: Survival of *Legionella* in windshield washer fluid

Survival of *Legionella* varied noticeably in fluids A, B, and C when prepared at half strength and incubated at 37 °C (Fig. 9). The initial concentration of 1.5×10^6 CFU/mL was maintained in the DI water and fluid A for 23 and 26 days, respectively. Thereafter, a steady decline in concentrations were recorded in the DI water and fluid A resulting in <1 CFU/mL at day 68 and 3.0×10^5 CFU/mL on day 73, respectively. In fluid B, the initial concentration was maintained for 7 days followed by a steeper decline than that observed for DI water and fluid A, with the *Legionella* population reaching <1 CFU/mL on day 42. In fluid C, the *Legionella* concentration declined to <1 CFU/mL by 1 day. In the DI water and fluid B, 3-log reductions in CFU/mL were observed by day 49 and 23, respectively. Only a half-log reduction in *Legionella* concentration was recorded in fluid A after 73 days. At 37 °C, the estimated T_{90} values for the DI water, fluid A, and fluid B were 33.36, 85.75, and 16.51 days, respectively.

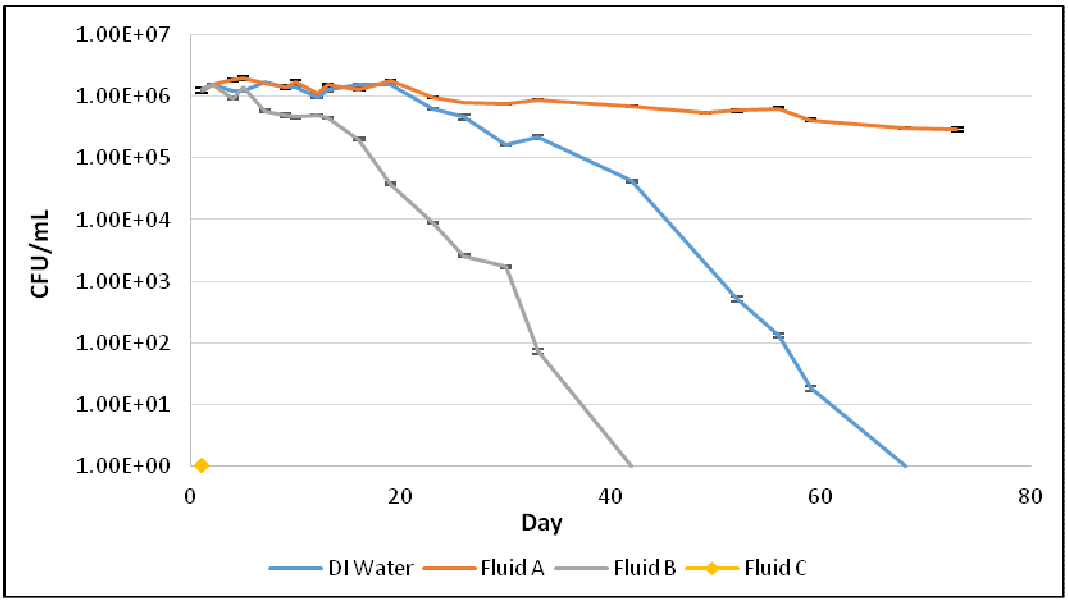

Fig. 9 - Survival of *L. pneumophila* in various fluids at 37 °C. Error bars represent standard error of duplicate samples.

Survival rates of *Legionella* at 25 °C were again dependent on the fluid the cultures were incubated in (Fig. 10). In the DI water and fluid A, concentration reduction over-time followed a similar trend for both throughout the study, in contrast to the trends observed at 37 °C. In both, a consistent and slow decrease was noted after 72 hours of incubation at 25 °C, with concentrations falling to 1.6×10^5 CFU/mL at day 70. *Legionella* concentration began decreasing in fluid B within the first 24 hours and reached <1 CFU/mL by day 23. The concentration in 10% methanol decreased drastically after day 1, resulting in a concentration of <1 CFU/mL by day 9. *Legionella* concentration in 20% methanol reached <1 CFU/mL by day 1. In fluid B and 10% methanol, a 3-log reduction in *Legionella* was recorded by day 18 and day 7, respectively; whereas, the DI water and fluid A demonstrated less than a log reduction after 70 days. At 25 °C, the estimated T_{90} values for the DI water, fluid A, fluid B, and

10% methanol were 72.67, 66.35, 8.00, and 4.61 days, respectively. Survival rates of *Legionella* at 25 °C were again dependent on the fluid the cultures were incubated in (Fig. 10). In the DI water and fluid A, concentration reduction over-time followed a similar trend for both throughout the study, in contrast to the trends observed at 37 °C. In both, a consistent and slow decrease was noted after 72 hours of incubation at 25 °C, with concentrations falling to 1.6×10^5 CFU/mL at day 70. *Legionella* concentration began decreasing in fluid B within the first 24 hours and reached <1 CFU/mL by day 23. The concentration in 10% methanol decreased drastically after day 1, resulting in a concentration of <1 CFU/mL by day 9. *Legionella* concentration in 20% methanol reached <1 CFU/mL by day 1. In fluid B and 10% methanol, a 3-log reduction in *Legionella* was recorded by day 18 and day 7, respectively; whereas, the DI water and fluid A demonstrated less than a log reduction after 70 days. At 25 °C, the estimated T_{90} values for the DI water, fluid A, fluid B, and 10% methanol were 72.67, 66.35, 8.00, and 4.61 days, respectively.

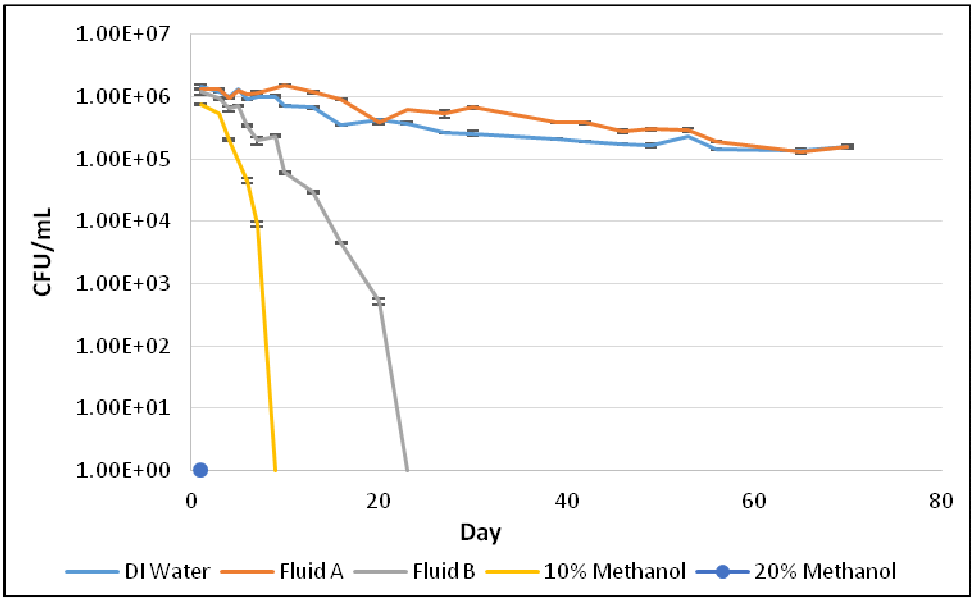

Fig. 10 - Survival of *L. pneumophila* in various fluids at 25 °C. Error bars represent standard error of duplicate samples.

4.4.2: Growth of *Legionella* in windshield washer fluid reservoirs

The concentration of *Legionella* in unspiked tap water incubated at 25 °C remained unchanged throughout the course of the experiment (Fig. 11), staying below the limit of detection for the culture based-assay used in the study. However, at 37 °C, the concentration started to increase by day 17, peaked at 1.9×10^3 CFU/mL by day 48, then steadily decreased until day 97. The concentration of *Legionella* in the spiked tap water incubated at 25 °C steadily decreased to 5.5×10^2 CFU/mL by day 38. In the spiked tap water incubated at 37 °C, the concentration increased and peaked at 9.8×10^3 CFU/mL by day 23, then decreased to 1.7 X 10^3 CFU/mL by day 41. On day 97, the concentrations in the spiked and unspiked tap water at 37 °C, and spiked tap water at 25 °C were 1.7×10^3, 4.7×10^2, and 3×10^2 CFU/mL, respectively. Additional samples were collected on day 215 and day 364. On day 215, the concentrations recorded were <1 and 5.8×10^2 for unspiked tap water at 25 °C and 37 °C, respectively. For the spiked samples the concentrations were <1 and 3.8×10^3 CFU/mL at 25 °C and 37 °C, respectively. By day 364, the samples maintained at 37 °C contained increased concentrations of 8.4×10^2 and 4.4×10^3 CFU/mL for unspiked and spiked tap water, respectively.

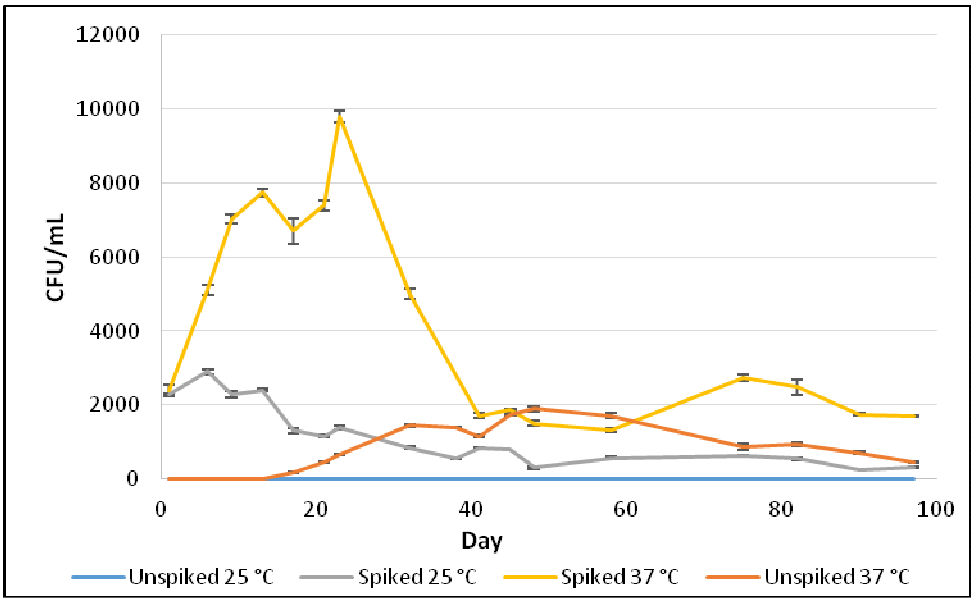

Fig. 11 - Growth of *Legionella* in windshield washer fluid reservoirs containing spiked and unspiked tap water at 25 and 37 °C. Error bars represent standard error of duplicate samples.

4.4.3: Environmental detection of *Legionella* in school bus windshield washer fluid

Ten out of the 12 buses (83%) contained culturable *Legionella* on at least one sampling event (Table 8). The drinking water fountain, sink faucet, and stock washer fluid solution each contained *Legionella* on at least one sampling event. Twenty out of 27 (74%) washer fluid samples collected contained culturable *Legionella* with concentrations ranging between 4.0 CFU/mL and 8.1×10^4 CFU/mL. Ten positive samples (37%) contained *Legionella* at concentrations higher than the stock washer solution (100 CFU/mL). Culturable *L. pneumophila* was confirmed by PCR in 8 samples collected from 6 buses, while *L. jordanis* was identified in 4 samples collected from 3 buses. Aerosolized *Legionella* was detected in 3 air samples from the windshield washer

spray of 2 buses, with concentrations ranging between 90 CFU/m^3 and 135 CFU/m^3. With the exception of fluid from bus 50, which was being maintained in an air conditioned garage at the time of sampling, all washer fluid temperatures recorded on sampling event 3 were higher than the air temperature of 38.0 °C, with a range of 38.7 °C to 41.4 °C.

High non-*Legionella* bacterial growth resulted in indeterminate quantification of *Legionella* in several washer fluid samples. It should be noted that heat treatment and the *Legionella* selective antibiotic cocktail used for media preparation substantially reduced the level and occurrence, but did not completely eliminate the growth of non-*Legionella* organisms in cultures from the field samples. All washer fluid samples negative for *Legionella* spp. by PCR also tested negative by the culture assay. One windshield washer fluid sample produced a negative PCR result for *L. pneumophila*, despite DNA extracted from an isolated colony cultured from this sample identifying it as *L. pneumophila*. Two washer fluid samples produced no detectable culturable *Legionella* (due to the presence of non-*Legionella* bacterial growth) but tested positive for the presence of *Legionella* DNA. Results for several PCR assays of washer fluid samples producing no culturable *Legionella* were indeterminate, potentially due to assay inhibition. Probable DNA extraction and/or PCR inhibition were observed for several samples, resulting in low PCR product concentration and quality. Spiked control tests were performed in which *L. pneumophila* cells or DNA were added to certain washer fluid samples and their extracts. These tests revealed inhibition occurring during the DNA extraction, purification, and amplification processes of the molecular analyses performed for specific washer fluid samples.

The engine temperature of a front engine bus running for 45 minutes was measured at 97 °C, while the washer fluid from this bus was 60 °C. Buses #6, 7, 9, and 11 were reportedly operated for 1-3 hours a day, 5 days a week, throughout the study. On sampling event 3, all buses sampled had been in use for 3 hours during the day prior to sampling. When activated, the bus windshield washers emitted fluid in bursts of approximately 15 mL.

	Sampling Event 1 Air Temp 38 ^{O}C		**Sampling Event 2 Air Temp 42 ^{O}C**				**Sampling Event 3 Air Temp 38 ^{O}C**				
Sample	CFU/mL	PCR	CFU/mL	PCR	Spp.	Air sample CFU/m^3	Sample Temp. (°C)	CFU/mL	PCR	Spp.	Air sample CFU/m^3
Sink Faucet	160	+	14	+	Lp	NA	30.5	10	+	Lp	NA
Drinking Fountain	ND	ND	100	+	ND	NA	22.0	0	-	ND	NA
Stock Solution	100	+	Ind	Ind	ND	NA	ND	ND	ND	ND	NA
Bus #1	ND	ND	120	+	Lj	ND	38.6	14	+	Lj	ND
Bus #2	ND	ND	4	+	Lj	ND	39.3	28	+	Lp	ND
Bus #3	0	+	60	+	ND	ND	41.4	6	+	Lj	ND
Bus #4	ND	ND	0	+	ND	ND	38.9	81,000	+	Lp	ND
Bus #5	ND	ND	1,840	+	Lp	ND	40.0	Ind	Ind	ND	ND
Bus #6	ND	ND	0	-	ND	ND	39.7	0	-	ND	ND
Bus #7	12,000	+	6,400	+	Lp	90	32.2	32,000	+	Lp	120
Bus #8	ND	ND	5,200	+	Lp	ND	39.2	12,400	+	Lp	135
Bus #9	ND	ND	340	+	ND	ND	38.8	1,280	+	Lp	ND

Bus #10	ND	ND	0	Ind	ND	ND	38.7	Ind	Ind	ND	ND
Bus #11	0	-	ND	ND	ND	ND	40.6	0	-	ND	ND
Bus #12	4	+	Ind	+	ND	0	39.0	Ind	Ind	ND	0

Table 8: Summary of *Legionella* data from windshield washer fluid, drinking water fountain, sink faucet, and air samples. NA: Not applicable, ND: No data collected, Ind: Indeterminate concentrations for plate count or indeterminate positivity for PCR assay. Lp: *Legionella pneumophila.* Lj: *Legionella jordanis*. PCR results indicate positive assay using *Legionella* genus primers.

4.5: Discussion

4.5.1: Survival of *Legionella* in windshield washer fluid

The low reduction in concentration of *Legionella* in fluid A during both experiments (Fig. 9 and 10) demonstrates the capability for the organism to persist in this particular washer fluid. The survival of *L. pneumophila* cultured at 25° C in fluid A, T_{90}=66.35 days, was similar to that in DI water, T_{90}=72.67 days, (p-value at day 70: 0.415) and significantly higher in half strength fluid A, T_{90}=85.75 days, than in DI water, T_{90}=33.36 days, (p value at day 59: 0.018) at 37° C. Two possible explanations for this are: 1) fluid A had a neutral or mildly antagonistic effect on *Legionella* survival yet contained nutrients to support growth (which would be absent in the DI water), or 2) fluid A, particularly at half strength, produced an environment more conducive to the survival of *Legionella* than that found in sterilized DI water. Regardless, the survival of *Legionella* in fluid A is highly relevant, not only for setting a precedent for the

pathogen's ability to maintain populations in washer fluid similar to fluid A (containing low concentrations of anti-freezing and cleaning agents), but also the potential for long term persistence in washer fluids.

The results for survival rates of *Legionella* in fluid B at both concentrations and both temperatures (Fig. 9 and 10), along with those measured with 10% methanol (Fig. 2), are interesting in that they highlight forms of washer fluid chemical compositions and concentrations in which *Legionella* can survive for a short period. By demonstrating some level of tolerance to a mixture of low concentration methanol, ethanol, and isopropanol (fluid B, T_{90} at 25°C=8.00 days), along with 10% methanol (T_{90}=4.61 days, found in washer fluids rated for just below freezing temperatures), these data highlight both the fact that *Legionella* may be capable of surviving to some extent in a wide variety of washer fluids, and that this survival may vary greatly between different fluids. This is demonstrated by the significant difference in survival observed for fluid A and B when incubated at 25°C (p-value at day 20: 0.039). Similar reasoning applied to the results showing complete reduction of viable cells when incubated in 20% methanol (found in washer fluids rated for temperatures above -15 °C) or fluid C (Fig. 9 and 10) leads to the assumption that certain washer fluids can effectively kill large concentrations of *Legionella* within a day. Testing a variety of washer fluids with varying anti-freezing and cleaning agents would be essential for determining ingredients and compositions capable of efficiently preventing the growth of *Legionella* or other pathogens in washer fluids.

The results from both survival experiments indicate that in certain washer fluids, and under the optimal conditions, *Legionella* could survive for extended periods of time.

Legionella can tolerate temperatures at 50 °C for extended periods of time (Dennis et al., 1984) and have been detected in a wide range of environments. The growth of this pathogen requires temperature in the range of 20 °C to 48 °C (Kusnetsoz et al., 1996, Schulze-Röbbecke et al., 1987) and a specific set of nutrients (Warren and Miller 1979), which can be extracted from endoparasitized host organisms (Moffet and Tompkins 1992) or microbes consumed via necrotrophy (Temmerman et al., 2006). While data from these survival experiments performed provide no definitive evidence for the growth of *Legionella*, it would not be unreasonable to assume that the temperature and nutrient conditions necessary for the growth of these organisms could feasibly occur within washer fluid reservoirs, particularly those containing windshield washer fluid prepared with tap water.

4.5.2: Growth of *Legionella* in windshield washer fluid reservoirs

The results summarized in Fig. 11 illustrate that *Legionella* are capable of growing, establishing, and maintaining a population in tap water added to a windshield washer fluid reservoir when incubated at 37 °C. Of particular interest is the increase in *Legionella* concentration seen in the reservoir containing unspiked tap water incubated at 37 °C. This increase from undetectable level to 2.0×10^3 CFU/mL demonstrate the potential for drastic increases in *Legionella* in stagnant tap water. In addition, the long term survival of *Legionella* spiked in tap water at 37 °C highlights the hardiness of this pathogen under the experimental conditions. In contrast to the survival experiments, the levels of *Legionella* in the reservoirs for both spiked and unspiked tap water (approximately 2500 CFU/mL and <1 CFU/mL, respectively) were relatively realistic, as

it would not be unexpected to find such concentrations in contaminated tap water. As 25 °C is near the low end of the growth range for *Legionella* (Schulze-Röbbecke et al., 1987), the lack of an increase in concentration over time was not unexpected. The difference in growth and long term survival at 25 °C and 37 °C could signify the importance of high ambient air temperatures (such as those recorded during the field study) for the growth of *Legionella* in washer fluid reservoirs.

4.3 Environmental Detection of *Legionella* in School Bus Windshield Washer Fluid

The results from the field study clearly demonstrate the potential for the presence of viable *Legionella*, including *L. pneumophila*, in windshield washer fluid within reservoirs. The high number of *Legionella* positive samples in the examined school buses suggests the contamination of washer fluid reservoirs could be a common phenomenon. *Legionella* contaminated tap water used in the preparation of washer fluid, low bactericidal properties of the washer fluid ingredients, and the high ambient temperatures in central Arizona are three potential factors encountered in the study are potential reasons for the high levels of contamination documented. The differences in the concentrations between the stock solution and the washer fluid in the reservoirs sampled, along with variation in concentration during different sampling events within the same reservoir, suggest *Legionella* growth had been occurring in the reservoirs. This may pose serious implications, namely that washer fluid reservoirs could serve as a source of amplification for *Legionella* to the point of posing a public health risk. Results from the air sampling performed in this study demonstrate that the aerosolization of high

concentrations of *Legionella* via the action of bus windshield washers may present a possible route of exposure not only for automobile drivers and passengers, but also for pedestrians and others in proximity to washer fluid spray.

In response to an epidemiological study performed by Wallensten et al. (2010), Palmer et al. (2012) investigated the prevalence of *L. pneumophila* in car windshield washer reservoirs in the United Kingdom and the organism's ability to grow in windshield washer fluid. In a field study of 30 vehicle washer fluid reservoirs with or without added "screenwash", only a single reservoir that had never contained "screenwash" sampled positive for *L. pneumophila* at a concentration of 6 CFU/mL, over four orders lower than the highest concentration we measured in school bus windshield washer fluid. In addition, Palmer et al. demonstrated the bactericidal capability of "standard" windshield washer fluid against *Legionella* at concentrations of 10 parts per million. While our study demonstrated certain washer fluids or components of washer fluids display bactericidal activity against *Legionella*, but not all examined washer fluids had this property; *Legionella* was able to survive in washer fluid A and sterile water to a similar degree. The discrepancies between the results produced by Palmer et al. and our own could largely be due to the differences in the climates between the two areas where the studies were conducted. Not only are the summer temperatures of 36 to 45 °C in Central Arizona close to ideal for *Legionella* growth (Kusnetsov et al., 1996), but potentially more important is the fact that windshield washer fluids designed for use in warm weather typically contains significantly lower levels of antifreezing agents such as methanol. Both studies, however, have produced evidence suggesting automobile washer

fluid reservoirs as a novel source for *Legionella* growth and possible transmission to humans.

4.6: Conclusions

- *Legionella* are capable of surviving for extended periods of time in certain automobile windshield washer fluids.
- Windshield washer fluid reservoirs can contain nutrients to support large *Legionella* populations.
- *Legionella* contamination in washer fluid reservoirs may be common.
- Aerosolization of *Legionella* in washer fluid spray is possible.

This is the first report of the long term survival of *Legionella* in washer fluid, viable *Legionella* in vehicle windshield washer fluid within reservoirs, and the emission of aerosolized *Legionella* from an automobile windshield washer. Our results suggest that the route of exposure to *Legionella* from automobile windshield washer fluid spray is a possibility. Further investigation on the growth and survival of *Legionella* in windshield washer fluids and reservoirs would certainly provide relevant insight into the contamination, transmission, and risk associated with this pathogen in this potential novel source of transmission.

CHAPTER 5

MALDI-TOF-MS CHARACTERIZATION OF *LEGIONELLA*

5.1: Abstract

As a water-borne environmental pathogen of increasing concern, techniques for cost-effective and rapid characterization of *Legionella* are vital, thus demonstrating the relevancy of research aimed at improving Matrix Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF-MS) analysis methodology for this microbe. First, optimal sample preparation methods for the analysis of environmental *Legionella* isolates via MALDI-TOF-MS were determined. These methods were implemented to perform strain-level characterization of environmental *Legionella* isolates from Central Arizona. Results demonstrate that a MALDI-TOF-MS method involving agar-based culturing and protein extraction-based sample preparation yield high quality mass spectra. Twenty-eight environmental *Legionella* isolates originating from two separate drinking water distribution systems were analyzed. Multiple species were detected and strain-level characterization was achieved, with 12 unique strains characterized. In addition, isolates of *L. pneumophila*, the most common species observed in the study, were correctly assigned to specific sampling sites. These results demonstrate the potential for this technique to be applied for sub-species characterization of bacteria, with significant benefits over established methodologies.

5.2: Introduction

Since the discovery of Legionnaires' disease in 1976, bacteria of the genus *Legionella*, particularly *L. pneumophila*, have become water-borne pathogens of significant concern, causing more drinking water related disease outbreaks than any other pathogen in the United States (Brunkard et al., 2011). Continually rising legionellosis incidence, as well as the variety of sources of transmission (Hicks et al., 2011), highlight the need for tools to aid in the rapid identification and characterization of these pathogens for public health purposes, such as tracking transmission sources during outbreak investigations. Due to the frequency with which these organisms are found in public water supplies and the low exposure event to disease rate, advances in high-resolution, rapid strain typing methodology are particularly relevant for *Legionella*.

The work of many studies has highlighted the potential of this technology and demonstrate that the ability to reliably type *Legionella* strains from various environmental water samples via MALDI-TOF-MS would have tremendous applicability to the medical, public health, and water industry (Ruelle et al., 2004). As an environmental pathogen of increasing concern, techniques for cost-effective and rapid characterization of *Legionella* are vital, thus demonstrating the relevancy of research aimed at improving MALDI-TOF-MS analysis methodology for this microbe. This goals of this study were twofold: 1) to optimize sample preparation methods for the analysis of environmental *Legionella* isolates via MALDI-TOF-MS, and 2) to implement these methods to perform strain-level typing on environmental *Legionella* isolates from Central Arizona, USA. Results demonstrate than an optimized MALDI-TOF-MS preparation method allowed rapid profiling of 28 environmental *Legionella* isolates, in which *L.*

pneumophila isolates were readily distinguished. In addition, sampling site-level and strain-level characterization were observed. By conducting a systematic and quantitative analysis of methodology optimization for the analysis of *Legionella* via MALDI-TOF-MS, followed by the utilization of this methodology to type several strains of this microbe originating from separate and varied environmental sampling sites, this study directly demonstrates the potential for the application of this technology in regards to the rapid characterization of this pathogen.

5.3: Materials and Methods

5.3.1: Environmental sampling

Environmental water samples of 50 to 1000 µL were collected from water originating from two drinking water distribution systems in central Arizona. Sampling site selection was based on the following criteria: drinking water systems in central Arizona, systems with different water sources, and systems separated spatially. In addition, one sampling site contained water with several unique properties, including: low/sporadic treatment, high salinity, sporadic fecal contamination, high levels of *Legionella* contamination. One system, located west of Phoenix, AZ, consisted of chlorinated ground water, while the other system, located east of Phoenix, AZ, consisted of conventionally treated chlorinated surface water. A total of 12 *Legionella* isolates from the west system were analyzed: 3 from tap water, and 9 from automobile washer fluid reservoirs filled with washer fluid prepared using the system's tap water. A total of 11 *Legionella* isolates from the east system were analyzed, all from tap water. All but

two samples derived from the east system were taken from tap water previously inoculated with a laboratory strain of *L. pneumophila* Knoxville-1 ATCC strain 33153 (American Type Culture Collection, Manassas, VA, USA). A stock culture of Knoxville-1 was also analyzed, alongside 4 isolates of Knoxville-1 sampled from laboratory experiments in sterile water. A total of 28 isolates were analyzed in this study, including one Knoxville-1 stock culture (K), 4 Knoxville-1 isolates (L1-4), 11 east environmental isolates (E1-11), and 12 west environmental isolates (W1-12) (Table 9).

Table 9: Isolates of *Legionella* used in the study

Isolate	K	L1-L4	E1-E5, E7, E8	E5, E6	E9-E11	W1, W5	W2-W4, W6-W9	W10, W11	W12
Water Source	Stock Culture	Laboratory Experiment	East System	East System	East System	West System	West System	West System	West System
Water Type	N/A	Tap Water	Tap Water	Tap Water	Tap Water	Tap Water	Washer Fluid	Washer Fluid	Tap Water
Spp.	Lp	Lp	Lp	Lp	Non-Lp	Lp	Lp	Non-Lp	Non-Lp

Isolate species were determined via PCR. Lp refers to *L. pneumophila* and non-Lp indicates species other than *L. pneumophila*.

5.3.2: *Legionella* culturing

All water samples were processed, and all *Legionella* isolates were cultured using previously described methods (CDC 2005). BD BBL Buffered Charcoal Yeast Agar (Diagnostic Systems, Sparks, MD, USA) media was used for *Legionella* culturing, with glycine, polymixin B, vancomycin, and cycloheximide supplemented for environmental

water samples. Water samples with low concentrations of *Legionella* were concentrated via membrane filtration using 0.45 micron cellulose filters (Millipore Corp., Bedford, MA). Environmental Water samples with high levels of background growth were subjected to heat treatment at 50° C for 30 minutes prior to plating (Wullings et al., 2011). All samples were cultured at 37° C for 72 hours, with an additional 96 hours if necessary for full colony formation. Colonies presumed to be *Legionella* based on morphology were simultaneously cultured onto BCYE and Tryptic Soy Agar (Diagnostic Systems, Sparks, MD, USA) for confirmation. Culture confirmed *Legionella* isolates were stored at -80° C in a long-term storage solution comprised of 75% Charcoal Yeast Extract Broth (CYE), 15% Glycerol, and 10% Millipore water (Millipore Corp., Bedford, MA). One liter of CYE media contained the following: activated carbon (2.0 g), yeast extract (10.0 g), ferric pyrophosphate (0.25 g), L-cysteine HCL (0.4 g), and distilled water (1000 mL).

5.3.3: DNA Extraction and Molecular Analysis

To determine if isolates belonged to the *pneumophila* species, PCR (Wullings et al., 2011) was performed. DNA extraction was performed on isolated colonies from environmental samples using a ZYMO Research yeast/bacterial DNA extraction kit (Zymo Research Corporation, Irvine, CA, USA). *L. pneumophila* specific *mip* gene primers LpneuF (5′-CCGATGCCACATCATTAGC-3′) and LpneuR (5′-CCAATTGAGCGCCACTCATAG-3′) were used. The PCR amplification mixture used consisted of: 12.5 µL Promega GoTaq Green MasterMix (Promega Biosciences LLC., San Luis Obispo, CA, USA), 10 µL DNA template, and 0.13 µM each primer, with a final reaction volume of 25 µL. Gel electrophoresis was performed in a 1% agarose gel

containing 0.05 µL/mL of 10,000X Invitrogen SYBR Safe DNA Gel Stain (Life Technologies Corporation, Carlsbad, CA, USA) to detect PCR products. Identification of isolates as *L. pneumophila* or non-*pneumophila* spp. is shown in Table 9.

5.3.4: MALDI-TOF-MS sample preparation

Isolates analyzed via MALDI-TOF-MS were prepared either from broth or agar cultures. For broth cultures, isolates were plated from storage onto BCYE and incubated for 72-120 hours. Isolated colonies were then inoculated into 5 mL of BCYE and incubated with shaking at 150 RPM for 72-120 hours. Cell density was normalized for all cultures to an optical density at 600 nm of 3 ± 0.2 OD units. The cultures were then centrifuged at 15,000 X g for 5 minutes, with the resulting supernatant decanted. After resuspending in 1 mL of Millipore water, a second centrifugation at 15,000 x g for 5 minutes was performed, followed by resuspension in Millipore water and a third centrifugation at 15,000 x g for 5 minutes. The supernatants was removed and the resulting pellets were subjected to further preparation. For agar cultures, isolates were plated from storage onto BCYE and incubated for 72-120 hours. Colonies were then removed from the plates and suspended in 1 mL of sterile Millipore water. Cell density was normalized to an optical density at 600 nm of 3 ± 0.2 OD units. The cultures were then centrifuged at 15,000 × g for 5 minutes, with the resulting supernatant decanted. The resulting supernatants were then removed and the resulting pellets were subjected to further preparation.

Two sample preparation methods were used for MALDI-TOF-MS analysis: intact cell (IC) and protein extraction (PE) preparations. For IC preparations, pellets were

resuspended in 100 µL of Millipore water by vortexing for 30 seconds. 100 µL of alpha-Cyano-4-hydrocinnamic acid matrix solution consisting of 50% acetonitrile (Sigma Chemical Company, Bedford, MA), 2.5% triflouroacetic acid (Alfa Aeser, Ward Hill, MA), and 47.5% Millipore water, with alpha-Cyano-4-hydrocinnamic acid added to saturation were added to the suspension followed by an additional 30 seconds of vortexing. 2 µL of the supernatant from this preparation were spotted to a 96-well ground steel target plate (Bruker Daltonics, Bilerica, MA) and allowed to air dry. For PE preparations, pellets were suspended in 25 µl of 70% formic acid (Avantor Performance Materials, Center Valley, PA), and vortexed for 30 seconds. Acetonitrile (25 µL) was then added to the suspension, followed by an additional 30 seconds of vortexing. The suspensions were then centrifuged at 15,000 x g for 5 minutes. 1 µL of the resulting supernatants were then spotted to a 96-well polished steel target plate and allowed to air dry. 1 µL of an alpha-Cyano-4-hydrocinnamic acid matrix solution was then overlayed onto each sample and allowed to air dry. Data from this study came from isolates cultures and prepared on three separate days. All preparations analyzed were spotted in triplicate. Both preparation methods effectively inactivated *Legionella* cultures, with complete reduction in culturable cells observed for both, allowing for the safe transport and handling of this pathogen during the study.

It should be noted that the standard methodology for performing MALDI-TOF-MS analysis on bacterial cultures calls for the use of a preparation of 1 mL of bacterial cells at an optical density of 1.0 OD units at 600 nm. Cultures at optical density of 3.0 OD units were used in this study to assure sufficient cell densities were achieved. To confirm the higher than standard number of cells used for the preparations in this study

did not compromise spectrum quality, a control test was performed comparing preparations from identical cultures prepared with the two optical densities.

5.3.5: MALDI-TOF-MS data acquisition and analysis

All samples were analyzed using a Bruker Microflex LRF MALDI-TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA) with a nitrogen laser (λ=337 nm) operating in positive linear mode. Calibration was performed prior to data collection using the following mass calibrants (Sigma Aldrich, St. Loius, MO): ACTH 1-17 (2094 Da), ACTH 18-36 (2466 Da), insulin oxidized B (3494 Da), insulin (5734 Da), Cytochrome C (12360 Da), and Myoglobin (16952 Da) suspended in matrix solution at a ratio of 1:1. FlexControl 3.0 software (Bruker Daltonics, Billerica, MA) was used to operate the instrument. Automatic data acquisition was performed for the samples using the following settings: laser power 25-75%, 2000-20000 Da range, peak evaluation with resolution higher than 100, random walk with 10 shots at raster spot, 300 satisfactory shots summed up in 100 shot steps.

Raw spectrum data were converted to text files using FlexAnalysis 3.0 (Bruker Daltonics, Billerica, MA) before preprocessing and further analyses, which were performed in BioNumerics 7.1 (Applied Maths, Austin, TX). Spectra were preprocessed in BioNumerics 7.1 using the software's default settings for relaxed peak detection. Baseline subtraction was performed using a rolling disc method, smoothing via a Kaiser Window filter, and peak detection via a continuous wavelet transform ridge algorithm with a signal to noise ratio of 5.

5.3.6: MALDI-TOF-MS optimization

To determine the optimum sample preparation and cell culturing methods for the environmental isolates used in the study, MALDI-TOF-MS data obtained from IC and PE sample preparations (with agar culturing) were compared for spectra quality, followed by samples prepared from agar and broth cultures (with PE sample preparation). For the agar/broth culture comparison, four environmental isolates were analyzed: two from the east system and two from the west system were compared alongside two stock cultures of Knoxville-1. For the IC/PE comparison, 13 environmental isolates were analyzed, 7 from the east system, and 6 from the west system, alongside two stock cultures of Knoxville-1. Identical data acquisition and analysis methods were applied to both sets of comparisons. The following parameters for peak quality were then determined for samples from the comparisons: base peak signal to noise ratio, base peak resolution, and peak range. Average values, along with their standard deviations, were calculated for triplicate samples of each culture condition or sample preparation method. Similarity matrices derived from cluster analysis were used to determine technical replicate reproducibility.

5.3.7: MALDI-TOF-MS cluster analysis

Cluster analysis was performed on spectra acquired from 34 cultures of the 26 isolates. All sample preparations used in this cluster analysis were prepared via agar/PEMS methods, determined to be optimum conditions for spectra quality. After preprocessing of raw spectra in Bionumerics 7.1, triplicate technical replicates of each isolate were summarized using the software's summary spectra function to generate one

composite mass spectrum (with a high level of stringency) for that isolate. Note that a single replicate from isolate L1 produced a spectrum with fewer than 5 peaks, which was suggested as low quality and, thus, was removed from the cluster analysis. For L1, only two technical replicates were summarized to generate the composite spectrum. Cluster analysis was performed using a complete linkage cluster analysis algorithm based on the Pearson correlation similarity coefficient. To determine the species level similarity percentage cut-off value in the cluster analysis performed, similarities between known *L. pneumophila* isolates (previously determined via PCR) were compared, with 32% similarity being chosen based on this rationale. A similar approach was taken to determine the strain level cut-off value of 90% similarity by comparing similarities of biological replicates of four isolates (Knoxville-1, W2, W5, and W10). A methodology for strain characterization similar to this has been performed previously (Pereira et al., 2013), in which strains of the fungus *Trichophyton rubrum* were distributed into sub-groups based on similarity values of 85% derived via cluster analysis of MALDI-TOF-MS spectra.

5.3.8: Statistical analysis

To determine whether the effects of the various preparation methods tested had a significant effect on the spectrum quality metrics examined, Student's T-tests were performed, with a p-value cut-off value of 0.05 used to determine significance using R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Jackknife analysis using average similarity was performed in Bionumerics 7.1 to quantify the ability of cluster analysis performed to correctly group isolates.

5.4: Results and discussion

5.4.1: MALDI-TOF-MS optimization

To evaluate sample culturing and preparation methods ideal for examining environmental isolates of *Legionella* via MALDI-TOF-MS, Agar/IC and Agar/PE preparation, as well as Agar/PE and Broth PE were compared. . Analyses of spectra quality for IC and PE preparations for three isolates, K, W5, and W11, were chosen for display due to their representation of the range of results observed in the study (Table 10). W5 had the most similar spectra quality using the two methods, while W11 had the most differing. Preliminary experiments comparing spectra quality parameters which were from agar and broth culturing methods have also been conducted. However some isolates did not produce quality spectra with broth/PE method (data not shown). Thus only quality of spectra obtained from agar culturing with different sample preparation methods has been compared in this study. Identical isolate preparations prepared from OD 1.0 and 3.0 cultures produced similar spectra and spectra quality parameters, particularly reproducibility, for both optical densities (Table 11 and Fig. 15).

Base peak signal to noise ratios were higher in PE preparation for all isolates with the exception of IC preparation for W5. Base peak resolution was higher in PE preparation for all isolates, although not significantly so for W5. Peak numbers were greater in IC preparations (substantially higher in broth culture) for all isolates. Reproducibility was higher for PE preparation for all isolates, although not significantly so for W5 (Table 2). It should be noted that certain isolates regularly produced spectra of

unacceptably low quality using broth culturing and IC preparation, occasionally to the point where data acquisition was impossible. For example, when prepared via broth culturing and PE preparation, only one of three replicate platings of W11 was able to be analyzed in FlexAnalysis.

Overall data of metrics for MALDI-TOF-MS spectra quality of *Legionella* comparing the two preparation and culturing methods suggest that agar culturing and PE preparation are the ideal. The universally higher reproducibility produced from this combination of methods reflects this. This difference is indicative of the superiority of these two methods for the *Legionella* isolates tested, for the purpose of high resolution MALDI-TOF-MS analysis. The reason for the higher reproducibility values seen from these methods may be attributed to the higher base peak signal to noise ratios, lower base peak mass variations, and greater base peak resolutions observed between triplicate samples of most isolates tested. These metrics of spectrum quality reflect the ability to distinguish background from sample, precisely measure peak mass, and distinguish unique peaks (Schumaker et al., 2012), all of which are of great importance for MALDI-TOF-MS analysis in general, but become increasingly so as the level of taxonomic resolution needed increases (Sedo et al., 2011). Refining MALDI-TOF-MS preparation and analysis methods to increase these metrics will most likely play a key role in the advancement of bacterial strain-level characterization via this technology.

Surprisingly, peak number, commonly used to determine spectra quality, appeared to be negatively correlated to reproducibility for most isolates analyzed. This may be attributed to the lower signal to noise ratios observed: analysis of samples prepared via IC preparation and broth culturing was less efficient at distinguishing background from

significant peaks, resulting in larger amounts of peaks. The relationship between reproducibility of this metric may indicate that its usefulness in measuring MALDI-TOF-MS spectra quality does not apply to all organisms or preparation methods, and could warrant further study. Interestingly, while agar culturing produced significantly higher quality spectra for all isolates tested than broth culturing, the disparities in quality metrics observed between IC and PE preparations varied between isolates. This is clearly seen in the reproducibility values (Table 10): the difference in reproducibility values between replicates of Knoville-1 for PE and IC preparation was 11.9%, for W5 it was 41.9%, and for W11, 3.7%. The variety of these isolates (W5 is a *L. pneumophila* strain unrelated to K, and W11 belongs to a separate species) most likely had an effect on the relative effectiveness of the two preparations to properly isolate and/or concentrate cellular components for analysis. It was noted during PE preparations that pelleted cells from certain environmental isolates showed more resistance to dissolving after application of formic acid and acetonitrile than others, including W5 and W11. This could be indicative of phenotypic variation, such as membrane composition, amongst *Legionella* environmental strains having an effect on sample preparation quality depending on the preparation method used and, thus, any MALDI-TOF-MS analysis based on data from these samples. In addition to this, environmental isolates of *Legionella* can have wildly different growth kinetics from well-established lab strains represented in varying nutritional requirements, increased incubation time, and reduced culturability (Buse et al., 2013). This variation on growth rates was observed amongst several environmental isolates used in this study, e.g., W11 produced mature colonies within 48 hr of culturing, whereas W12 took up to 120 hr. While the importance of organism-dependent MALDI-

TOF-MS methods selection has been established (Kern et al., 2013), results from this study further highlights this significance of methods optimization for the characterization of not only species, but also strains of *Legionella* and, potentially, other microbes.

To date, the majority of studies on *Legionella* analysis via MALDI-TOF-MS utilized simple smear preparations (Gaia, t al., 2012, Moliner et al., 2010, Svarrer et al., 2011, Pennenac et al., 2012), a method which sacrifices sample quality for lower preparation time and cost. In a study to determine optimal protein extraction preparation methods for inactivating pathogenic bacteria, Drevinek et al, (2012) determined that the use of ethanol and formic acid versus other solvents resulted in a greater number of higher intensity peaks for *Legionella* samples. In their study focused on typing environmental *Legionella*, Fujinami et al. (2010) employed two protein extraction methods: 1) a vortex method involving the use of TFA and a 0.2 µm filter to isolate proteins, and 2) a method involving the use of a bead beater on cell suspensions frozen in liquid nitrogen, with the former proving superior for use in MALDI-TOF-MS analysis. The improved quality of spectra generated by the use of optimized methods *Legionella* isolates, particularly protein extraction preparation, from our own and others' studies demonstrates the importance of utilizing ideal methodology for high-resolution characterization of this organism via MALDI-TOF-MS.

Table 10: Comparisons of spectra quality parameters in IC vs PE preparation for three isolates of *Legionella*.

Sample	Base Peak S:N Ratio	Base Peak Resolution	Peak Number	Reproducibility (%)

K IC	364.4±275.2	627.0±30.2	94.7±156.2	80.9±6.5
K PE	480.9±219.0	647.6±35.0	23±2.6	92.8±4.7
W5 IC	616.9±657.7	650.2.1±31.1	120.0±79.2	83.5±9.6
W5 PE	327.1±188.9	655.3±27.5	33.3±0.6	87.2±8.8
W11 IC	103.0±44.4	742.1±47.7	444.3±275.2	50.0±23.7
W11 PE	275.4±24.9	782.8±68.8	104.0±32.9	91.9±1.2
Avg IC	361.4±256.9	673.1±60.9	219.7±194.9	71.5±18.6
Avg PE	361.1±106.9	695.2±75.9	53.4±44.1	90.7±2.9

Data shown represent averages of triplicate samples from a single preparation followed by their standard deviations. Results for all parameters show a significant difference between IC and PE with the exception of W5 base peak resolution and reproducibility.

5.4.2: MALDI-TOF-MS cluster analysis

Cluster analysis was performed on samples of *Legionella* isolates prepared via methods previously determined to be optimum (PE preparation from agar cultures), with the goal of achieving strain-level characterization. Fig. 12 displays the dendrogram constructed via cluster analysis of the 28 analyzed isolates, along with each culture's respective summary spectra, represented in a pseudo-gel view. Branches for each culture are color-coded based on presumptive strains predicted using a 90% similarity cut-off. After the species/strain code is the name of the isolate and date of data acquisition MDS was performed to better visualize the relationships between the analyzed isolates. Fig. 13 displays the results of the cluster analysis in the form of an MDS scatterplot, carrying over the color-coding assigned in the dendrogram of Fig. 12.

Using the species and strain cut-off limit of 32 and 90% similarity, respectively, the cluster analysis revealed 4 non-*pneumophila* species and 14 strains of *Legionella* amongst the 28 isolates examined. All 8 Knoxville-1 cultures and 5 isolates (including the stock culture) clustered closely and distinctly, with an average similarity of approximately 93%. Replicate cultures of the 6 *L. pneumophila* East isolates formed three strains, while the 9 *L. pneumophila* West isolates formed 4 strains. The 5 East and 3 West non-*pneumophila* isolates formed 4 separate species, two of which had two distinct strains amongst their isolates. *L. pneumophila* environmental isolates (with the exception of W9, which grouped close to Knoxville-1) formed distinct clusters based on sampling site. Five West *L. pneumophila* isolates formed a single strain, with an average similarity of 94.6%. No replicate isolates' cultures clustered apart and no *L. pneumophila,* and non-*pneumophila* isolates clustered together. Fig. 13 depicts definitive, near equidistant, separation of isolates by species, along with clustering of strains and species in accordance assigned via cluster analysis similarity, with the possible exception of isolates belonging to LpS1 and LpS2 (isolates E1-E4), which appear to be evenly spaced amongst themselves. Jackknife analysis resulted in 100% rates of correct classification for all presumptive strains, with the exception of LpS1 and LpS2, which incorrectly classified as each other at a rate of 50%.

The clearly defined clustering of isolates depicted in figures 12 and 13, including those of *L. pneumophila* by sampling site, suggest that the cluster analysis performed on MALDI-TOF-MS spectra of environmental *Legionella* isolates was able to characterize these isolates to the strain level. These results are validated by several factors. The high similarity values amongst multiple Knoxville-1 cultures and isolates, as well as replicate

cultures of environmental *Legionella*, demonstrate an appropriate level of accuracy and precision in sample preparation, data acquisition, and data analysis methods performed in the study. The lack of incorrect clustering between known *pneumophila* and non-*pneumophila* isolates suggests a high level of accuracy for the method in differentiation of these organisms at the species level. The agreement in clustering between Fig. 12 and Fig. 13, as well results from the jackknife analysis (with the notable exception of strains LpS1 and LpS2), show a reasonable level of confidence in the predicted strains. This is demonstrated particularly well for isolates of strains Knoxville-1 and LpS5, both of which clustered tightly and distinctly amongst themselves, despite the presence of other relatively similar strains (W9 and W8, respectively).

This study has yielded the following novel results: strain level profiling of *Legionella* isolated from tap water and belonging to multiple species, typing of the greatest number of *Legionella* strains in a single study, and sampling site-dependent typing of several strains of *Legionella*. Differentiation of the 5 *Legionella* species was reliably achieved, regardless of clustering approaches (data not shown) or preparation and culturing methods, which were optimized for the strain typing results generated. This helps to explain why species level characterization has been well documented for *Legionella*, including environmental isolates, (Gaia et al., 2011, Moliner et al., 2010), but strain-level profiling of these bacteria has only been reported once (Fujinami et al., 2011). The level of similarity and tight clustering between isolates W3-7 not only suggests a high level of resolution for the analysis used, but also demonstrates the potential for applied typing of environmental *Legionella*. Isolates W3, W4, W6, and W7 were cultured from automobile washer fluid in four separate vehicles prepared using tap water

originating from the same faucet that produced the tap water isolate W5 was cultured from. Even more significant is the fact that three additional isolates from separate vehicles, W1, W8, and W9, along with W2, a second isolate from the same tap water as W5, all belonged to strains distinct from W3-W7. The fact that multiple strains of *L. pneumophila* originating from the same source were able to be distinguished suggests that MALDI-TOF-MS could be used in a variety of applications, such as tracking the source of contamination of a *Legionella* strain responsible for an outbreak of Legionnaires' disease.

The fact that isolate W9 clustered so closely to isolates of Knoxville-1 is interesting. In addition to spectra appearing qualitatively different between the isolates (Fig. 14), in depth analysis of spectra from W9 isolate (data not shown) revealed it to be definitively distinct from Knoxville-1, possessing peaks not seen in Knoxville-1 (i.e. at 9546 Da), while lacking peaks found in all Knoxville-1 isolates (i.e. at 10386 Da). These results would seem to indicate that W9 is incidentally an environmental strain of *L. pneumophila* more closely related to the type strain Knoxville-1 than to the other environmental strains isolated from the same source in this study. Several strain and species designations were assigned in this study were not as clearly definitive as others, including the strain separations of LpS1/LpS2, LpS5/LpS6, and Spp2S1/Spp2S1, as well as the species separation of Spp2/Spp3. While all of these separations were assigned due to their respective isolates falling below the cluster analysis similarity cut-off values determined from known strains and species, the isolates in question were all within 5% of the requisite similarity for same species/strain designation (1.2% for LpS1/LpS2). The three dimensional view of the relationships between isolates in Fig. 2 suggest that distinct

clustering and, thus, potential strain/species distinction, did occur for these isolates, with the possible exception of LpS1/LpS2. The four isolates belonging to these assigned strains appear to be relatively evenly spaced amongst themselves, suggesting they may form a single distinct strain, albeit with isolates relatively less related when compared to those of the other presumptive strains of the study. This idea is further supported by the 50% rate of correct classification between LpS1/determined via jackknife analysis. The potential single strain status of these four isolates indicates that they may be an example of a group of *Legionella* near the limit of detection for strain level profiling using the methods performed, although higher resolution spectra data generated through improved methodology may result in these distinct strain separation amongst them.

In addition to continuing the optimization of methodology for analysis of *Legionella* via MALDI-TOF MS, two main goals will be addressed in future works. First, analysis of more environmental isolates from additional and previously sampled sites will be performed. By examining isolates from additional sources, sample types (e.g. soil, wastewater, etc.), and across greater geographical space, the true extent of the practicality of methodology developed will be validated. Repeated sampling from similar sites could generate compelling results on the temporal effects of *Legionella* contamination in regards to the strain distribution dynamics of the microbe. In addition to characterization of additional isolates, comparison of the use of MALDI-TOF MS to conventional typing methods typing for *Legionella*, namely PCR, will be performed to validate the accuracy of the developed methodology.

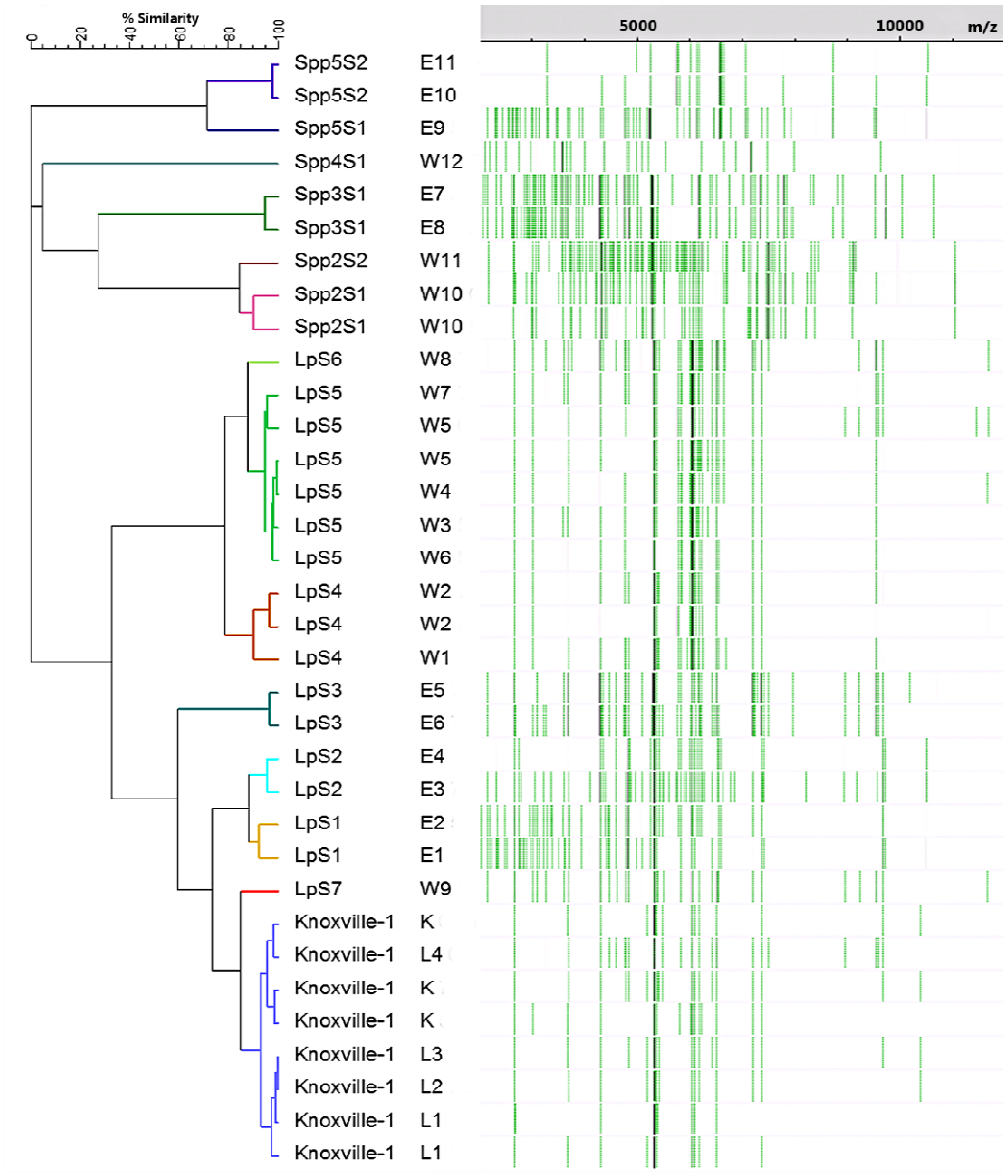

Fig. 12 – Dendrogram created via cluster analysis of summarized mass spectra from three technical replicates of 34 cultures of 28 *Legionella* isolates, along with respective mass spectra band data. Similarity coefficients were calculated using peak-based Pearson correlation and clustering was performed via complete linkage clustering. Cultures K and L1-L4 are isolates of the lab strain Knoxville-1. Samples E1-11 and W1-W12 are environmental isolates from east and west central AZ, respectively. Environmental isolates are labeled as members of *L. pneumophila* (Lp)

or other *Legionella* species (Spp) followed by presumptive strain group numbers (e.g. S1) based on the analysis. Terminal branches are color-coded based on presumptive strain groups.

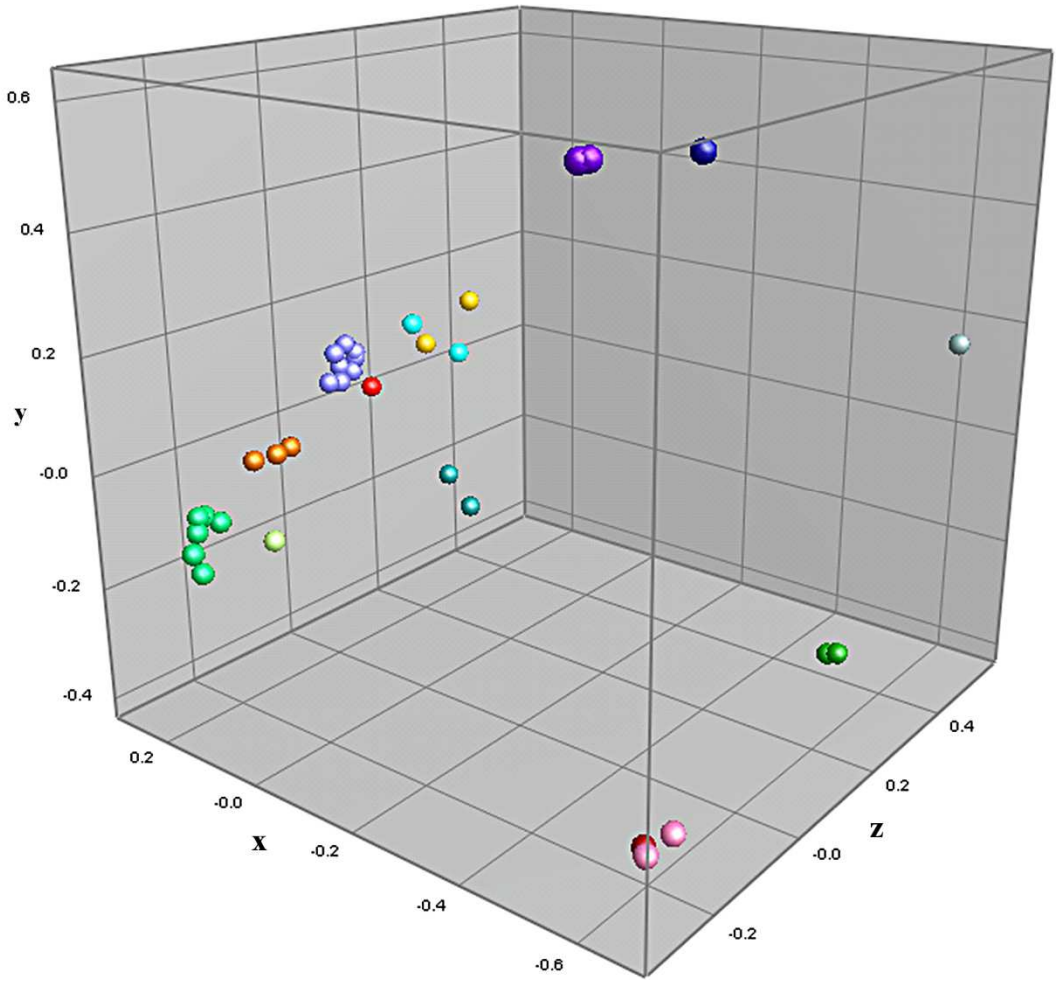

Fig. 13 – MDS scatterplot, with optimized positioning, based on cluster analysis described in Fig 12. Data points are color-coded based on presumptive strain groups.

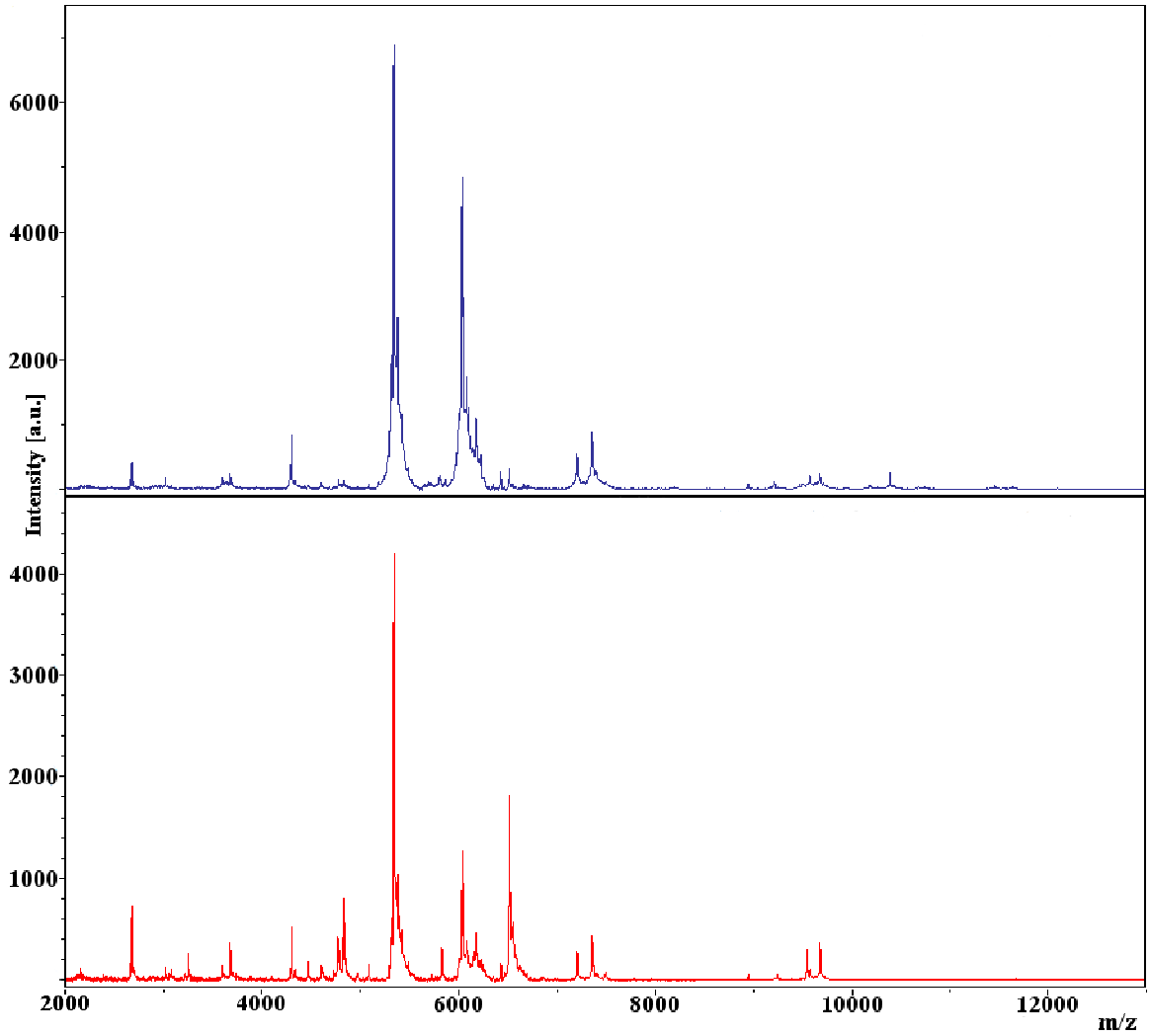

Fig. 14 – Example spectra generated from samples of isolates of Knoxville-1 (top) and W9 (bottom).

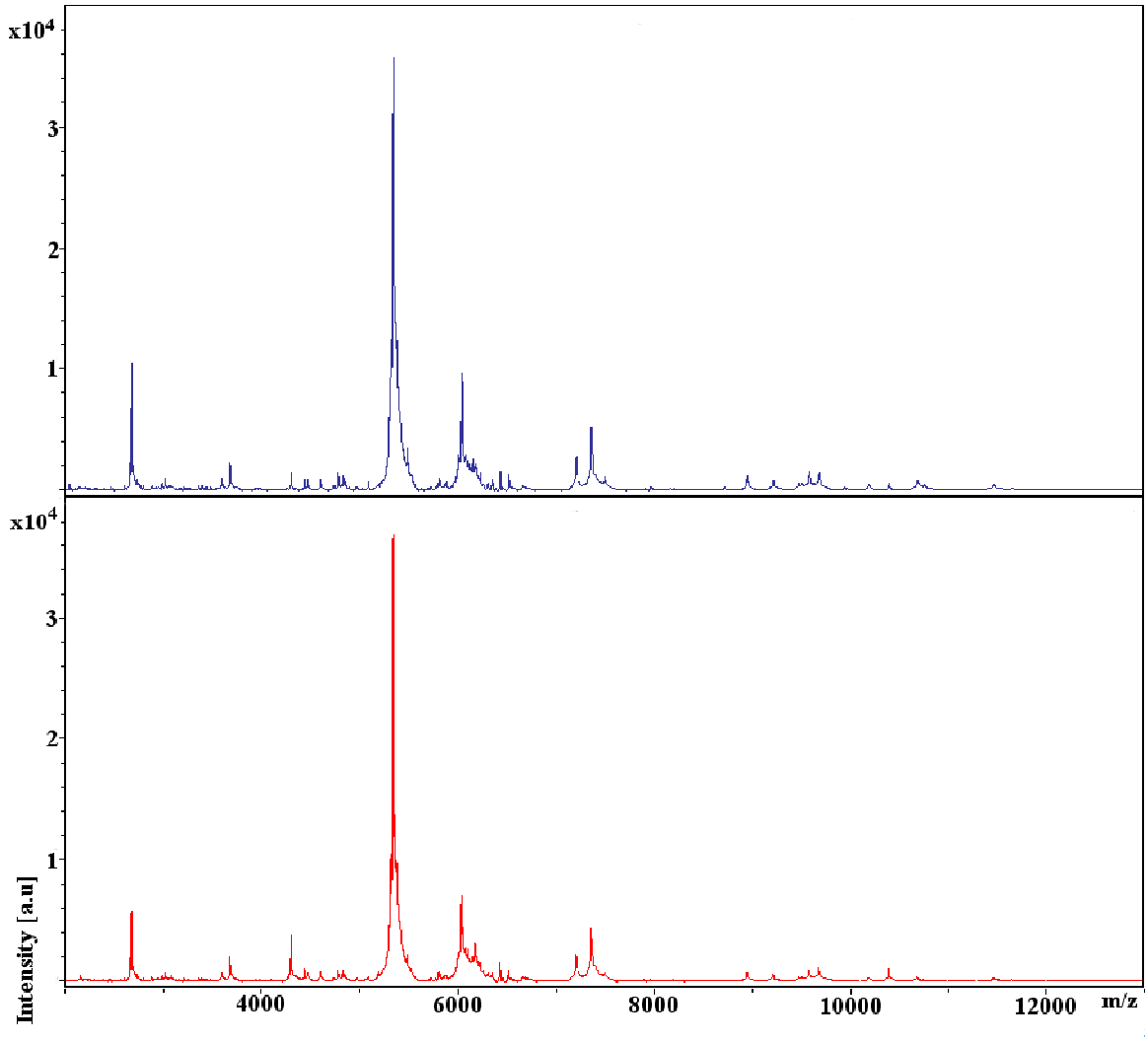

Fig. 15 – Example spectra generated from samples of strain Knoxville-1 prepared from cultures of optical density of 3.0 (top) and 1.0 (bottom) OD units at 600 nm.

Table 11 – Spectra quality data from samples of strain Knoxville-1 prepared via cultures of optical density of 3.0 (top) and 1.0 (bottom) OD units at 600 nm.

Sample	Base Peak S:N Ratio	Base Peak Resolution	Peak Number	Reproducibility (%)
OD 3.0	1173.2±403.5	645.8±42.6	29.3±6.5	96.6±1.7
OD 1.0	1477.6±358.3	646.3±4.6	34.7±13.3	96.4±1.1

Data shown represent averages of triplicate samples from a single preparation followed by their standard deviations.

5.5: Conclusions

- A universally ideal methodology for bacterial characterization via MALDI-TOF-MS may not be possible.
- Optimization of sample preparation can vastly improves results for MALDI-TOF-MS bacterial analysis.
- Strain level typing of *Legionella* via MALDI-TOF-MS is possible.
- Benefits of the MALDI-TOF-MS based typing make it promising for certain applications.

Through the systematic optimization of sample preparation methodology, strain level profiling of both *L. pneumophila* and non-*pneumophila Legionella* species was achieved. Results from this study have implications for a number of fields, including public health, environmental engineering, and microbial ecology, not only for *Legionella*, but other environmental microbes as well: the ability to reliably type microbes isolated from various environmental sources in a cost-efficient and timely manner would be greatly beneficial to those interested in tracking disease progression, monitoring drinking

water, or studying microbial population dynamics. As resolution of MALDI-TOF-MS profiling of microbes continues to improve, the use of this technology will undoubtedly be investigated for further commercial and academic applications, warranting further research into improving its use in typing beyond the species level. For *Legionella* in particular, further improvement of methodology, analysis of an increased number of environment and type-strain isolates, and comparison to established typing methods, will help to further research in this area.

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